Proceedings of the Workshop on Lipoprotein Heterogeneity

U.S. DEPARTMENT
OF HEALTH AND
HUMAN SERVICES

Public Health Service

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PREFACE

During the past several years the heterogeneity of the lipoprotein classes traditionally separated by ultracentrifugation (chylomicrons, VLDL, IDL, LDL and HDL) has become evident. All of these lipoproteins classes have been further fractionated into subclasses using gradient gel electrophoresis, immunoaffinity column chromatography and a wide variety of other techniques. The subclasses have also been shown in many instances to be a heterogeneous collection of particles with distinct physical characteristics, chemical composition and immunological properties. However, in most instances the origin, metabolism and atherogenicity of these subclasses are presently unknown.

The Lipoprotein Heterogeneity Workshop was conceived by the Atherosclerosis, Hypertension and Lipid Metabolism Advisory Committee and implemented by the Lipid Metabolism Atherogenesis Branch of the National Heart, Lung, and Blood Institute (NHLBI) to address several questions, including:

1. What evidence is there for structural, functional and metabolic (kinetic) heterogeneity of plasma lipoprotein classes and subpopulations?

2. What are the advantages and limitations of the methods currently used for the identification, separation and measurement of lipoprotein subpopulations?

3. What are the physiological and pathophysiological consequences of lipoprotein structural, functional and kinetic heterogeneity?

4. What measures, if any, should be undertaken by the NHLBI to facilitate ongoing or future research in the area of lipoprotein heterogeneity?

The workshop was organized into nine sessions each followed by a brief discussion period plus a tenth Summary Discussion period.

Special thanks are due to Drs. Petar Alaupovic, John Albers, Bryan Brewer, Waldo Fisher, Sandra Gianturco, Ronald Krauss and Gustav Schonfeld who helped formulate the agenda; and also to Drs. Sandra Gianturco, Alan Fogelman, Paul Nestel, Scott Grundy, Waldo Fisher, Alan Chait, John Albers, and Paul Roheim who chaired the sessions of the workshop and summarized their individual sessions (Arteriosclerosis 7, No. 3, May/June 1987).

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Workshop on Lipoprotein Heterogeneity

Sponsored by the National Heart, Lung, and Blood Institute

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OBJECTIVES OF THE WORKSHOP

1. To bring together a group of experts who will discuss the present state of the art relevant to methods used for the separation and quantitation of lipoprotein subclasses.

2. To produce a written document for the National Heart, Lung and Blood Institute summarizing the technological limitations of and unresolved questions related to separation and measurement of lipoprotein subclasses as well as the clinical significance of these measurements.

3. To distribute this document to basic and clinical researchers in order to make them more aware of the advantages and disadvantages of currently used separation and analytical techniques.

4. To discuss the need for the National Heart, Lung and Blood Institute to foster additional research in the area of lipoprotein subclass separation and quantitation; to promote efforts toward the standardization of lipoprotein subclass nomenclature; or to assist in making monoclonal antibodies or other reagents used to assay apolipoproteins more generally available to the research community.

INTRODUCTORY REMARKS

Kenneth Lippel, Ph.D.

I would like to welcome all of you on behalf of the National Heart, Lung and Blood Institute at NIH to this workshop on lipoprotein heterogeneity. A special welcome is extended to our colleagues from Australia, Finland, Scotland and Canada. I also want to thank the Planning Committee, Drs. Gianturco, Alaupovic, Albers, Brewer, Fisher, Krauss and Schonfeld for helping to formulate the workshop agenda. I also wish to thank the several companies whose financial support helped pay for some of the workshop expenses.

During the past few years, there has been a renewed interest in lipoprotein heterogeneity. All of the traditional lipoprotein classes, chylomicrons, VLDL, LDL, and HDL, initially separated by density centrifugation have now been further separated into subclasses by immunoaffinity chromatography and a wide variety of other techniques.

Numerous studies have shown that each class of lipoproteins is a heterogeneous collection of particles with distinct physical characteristics, chemical composition, and immunological properties. However, in many instances, the origin, the metabolism, the physiological significance of these subpopulations are unknown. Hopefully, the workshop will address some of the following unanswered questions:

1. What are the advantages and limitations of the methods being used to separate and quantitate the lipoprotein subclasses?

2. How do the lipoprotein subclasses separated by each method compare and contrast to each other?

3. What is the current state of knowledge of lipoprotein subclass metabolism and how is this metabolism related to atherogenesis and the risk of coronary heart disease?

Each presentation has been allotted 25 minutes which, if time permits, includes five minutes for questions and answers. In addition, each session includes a separate 15-minute discussion period in which everyone present is encouraged to participate.

Wednesday morning we will have a panel discussion and summarize our two and a half days of presentations. At that time, we will welcome recommendations concerning future research or other measures that the NHLBI should undertake to clarify the significance of lipoprotein heterogeneity.

SESSION I. Isolation, Origin, and Processing of ApoB-Containing Lipoproteins

Chair: Sandra H. Gianturco

PHYSICAL HETEROGENEITY OF APOLIPOPROTEIN B-CONTAINING LIPOPROTEINS

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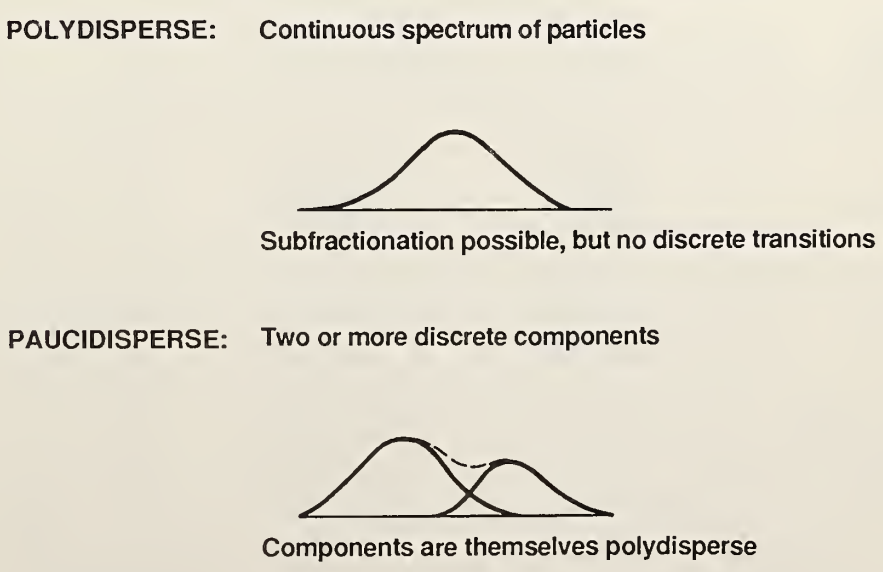
Introduction

The major physical properties that contribute to heterogeneity of apolipoprotein B (apoB)-containing lipoproteins include buoyant density, size, and shape. Hydrodynamic behavior of lipoproteins is influenced by each of these properties, and thus ultracentrifugation has been one of the most informative methods of assessing the physical heterogeneity of the very low to low density lipoprotein spectrum. The techniques employed include analytical (1,2), zonal (3), cumulative flotation (4,5) and density gradient ultracentrifugation (2,6-9). In general, lipoprotein solutions studied by these methods behave as polydisperse systems, i.e., broad distributions consisting of an indefinite number of components (Figure 1). Hence with standardized analytic ultracentrifugation (1), broad unimodal schlieren spectra are usually observed for low density lipoproteins (LDL) of flotation rate (S_f°) 0-12, for very low density lipoproteins (VLDL) of S_f° 20-400, and often for intermediate density lipoprotein (IDL) of S_f° 12-20. Similar broad and apparently continuous distributions are usually found with zonal ultracentrifugation (3), as well as with gel filtration chromatography (10). In this review, recent studies will be summarized which indicate that each of these major lipoprotein classes consist of a relatively small number of discrete components. This type of multicomponent heterogeneity is designated paucidispersity (11), although each of the individual components in the system exhibits some degree of polydispersity (Figure 1).

FIGURE 1

TYPES OF ULTRACENTRIFUGAL HETEROGENEITY


POLYDISPERSE: Continuous spectrum of particles
Subfractionation possible, but no discrete transitions
PAUCIDISPERSE: Two or more discrete components
Components are themselves polydisperse

LDL Subspecies

A number of investigators have employed ultracentrifugal methods to characterize heterogeneity of LDL (2,6-8,12,13). Multiple peaks in the schlieren profiles of S_f° 0-20 lipoproteins were first demonstrated by Hammond and Fisher in patients with hypertriglyceridemia (2). Such multiple peak patterns were also found in 9% of normolipidemic subjects (14). More recently, equilibrium density ultracentrifugation was employed in our laboratory to assess LDL heterogeneity in normal subjects (6,7). Distinct isopycnic bands were observed in most subjects, and these could be grouped into four major density groups, which have been designated LDL-I - LDL-IV (Table 1). In most normal subjects, however, one or two major bands are observed, with secondary peaks or shoulders corresponding to one or more of the other components (7). Further evidence for the discrete nature of these components has been provided by non-denaturing gradient gel electrophoresis, which separates lipoprotein particles on the basis of differences in size and shape (7). LDL analyzed by this technique in individual subjects exhibit up to 4-5 distinct bands (Figure 2).

While there is, in general, a correspondence of size and density of LDL, intra- and inter-individual variation in apparent size of LDL species of similar density has led to the identification of a larger number of LDL subspecies than can be discriminated by density banding (7). Since ultracentrifugal flotation rate is related to both density and size of LDL particles (6,7,15), characteristic peak S_f° rates are found for each major LDL component. Physical properties of six major LDL subpopulations are summarized in Table 1. When Lp(a) is found in the LDL density region,it may overlap with LDL-IV (7).

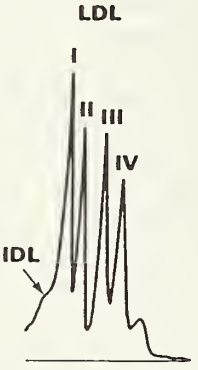


FIGURE 2: Multiple components in the d 1.019-1.063 g/ml lipoprotein fraction from a normal female subject (7,16), as demonstrated by non-denaturing 2-16% gradient gel electrophoresis.

Table 1. Major subspecies of Plasma LDL*

Species	Density Peak (g/ml)	Particle Diameter (Å)**	Peak S_f°
LDL-I	1.025-1.035	260-275	7.5-11
LDL-II	1.032-1.038	255-270	5.5- 8
LDL-IIIA	1.038-1.050	247-252	5 - 6
LDL-IIIB		242-246	3 - 5
LDL-IVA	1.048-1.065	233-242	0 - 4
LDL-IVB		218-232	

* From Reference 16.
** As determined by gradient gel electrophoresis.

In most normal subjects, the subspecies of greatest abundance is LDL-II. Women tend to have more mass of LDL-I than men, while men tend to have more LDL-IIIA than women (6,17). Recently, we have found that 25-30% of normal subjects aged 40 and older have an LDL subspecies pattern with predominance of LDL-III (18). Initial studies in families have indicated that this pattern is influenced by a single major genetic locus, and is associated with relative increases of VLDL and IDL and reduction in HDL_2 (18). These lipoprotein characteristics, including predominance of LDL-III, are shared by patients with familial combined hyperlipidemia (19), suggesting an association of this disease entity with a relatively common lipoprotein phenotype. In patients with severe hypertriglyceridemia, on the other hand, there is a relative predominance of denser LDL species, particularly LDL-IV, consistent with the earlier findings of Hammond and Fisher (2). Reversal of this pattern has been reported in conjunction with therapeutic lowering of triglyceride levels (20).

VLDL Subspecies

Ultracentrifugal techniques generally have not identified discontinuities within the VLDL spectrum except for the β-VLDL peak seen in dysbetalipoproteinemia (Type III hyperlipoproteinemia) (21). However, subfractions of differing physical and chemical properties have been isolated by a variety of physical techniques, including cumulative flotation (4,5), zonal ultracentrifugation (3), gel filtration chromatography (10), and nonequilibrium density gradient ultracentrifugation (9). Despite the differing methodologies employed, the use of normal and hyperlipidemic subjects, and the likelihood of varying sources of technical artifact, consistencies in physical and chemical properties of three major VLDL components are observed when appropriate fractions isolated by each of these techniques are compared (Table 2). While VLDL-I and -II have not been shown to be physically distinct, non-denaturing gradient gel electrophoresis has revealed two discrete bands with sizes corresponding to VLDL-II and VLDL-III (ref. 9, where these species are referred to as large and small VLDL, respectively).

Table 2. Properties of VLDL Subfractions separated by four methods*

Subfraction			% Composition (w/w) Prot.	Free Chol.	Chol. Ester	Phos.	Trig.	Avg. Size (nm)	Peak S_f°
I	B	CF	10	2	8	15	66	53	150
	2	ZO	8	5	10	13	64	--	139
	III	COL	8	5	8	13	77	50	---
II	C	CF	12	3	9	17	60	42	85
	4	ZO	9	7	14	16	54	--	68
	IV	COL	10	5	9	16	60	40	--
	1	DGU	11	6	8	17	58	40	~80
III	D	CF	15	6	18	19	42	32	37
	6	ZO	12	8	19	18	44	--	41
	VI	COL	15	6	16	19	45	30	--
	2	DGU	17	7	23	22	43	33	--

* Codes: CF = cumulative flotation (5); ZO = zonal ultracentrifugation (3); COL = column chromatography (10); DGU = single-spin density gradient ultracentrifugation (9).

IDL Subspecies

Gradient gel electrophoresis of subfractions obtained by nonequilibrium density gradient ultracentrifugation of d<1.030 g/ml lipoproteins (9) has revealed multiple components in the density interval commonly used to define IDL (1.006-1.019 g/ml). Two major size subpopulations have been identified, designated here as IDL-I and IDL-II. Physical characteristics of these species are given in Table 3.

Table 3. Properties of IDL Subspecies*

	Peak Density Range (g/ml)	Particle Diameter (Å)**	Peak S_f°
IDL-I	1.008-1.022	280-300	14-20
IDL-II	1.013-1.028	270-285	10-16

* From References 9 and 16.
** Gradient gel electrophoresis.

The larger triglyceride-enriched IDL-I component in most cases forms a continuous distribution with VLDL-III. Similarly, the smaller, relatively cholesterol-enriched IDL-II species usually appears to form a continuous distribution with LDL-I. Figure 3 presents a scheme for defining the major components within the S_f° 0-400 range based on the findings summarized above.

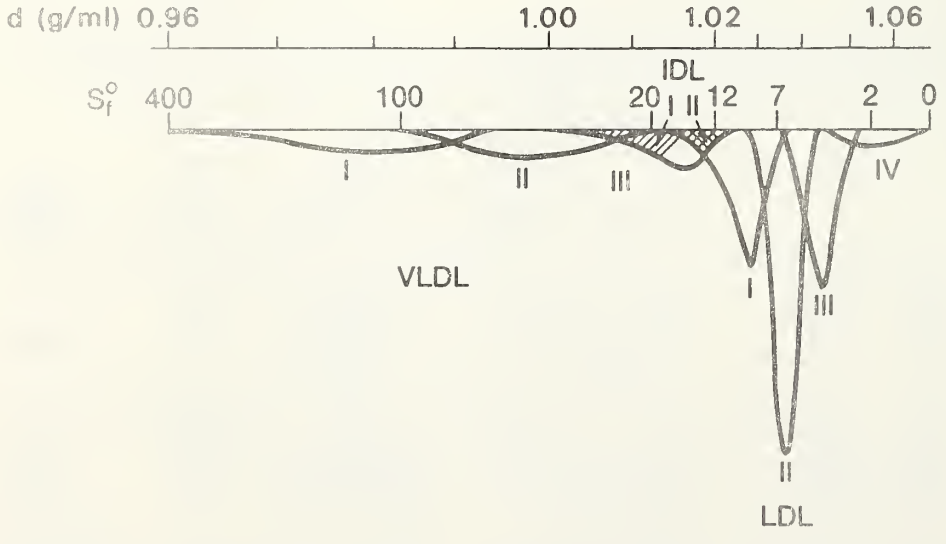
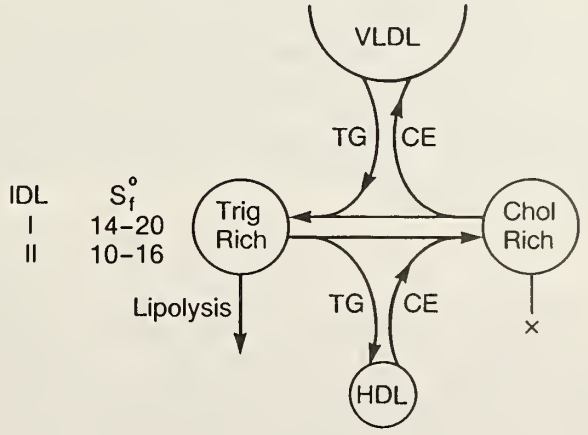


FIGURE 3: Major components within the VLDL, IDL, and LDL density intervals. Individual components are depicted by analytic ultracentrifugation schlieren curves with properties based on those listed in Tables 1-3. Total mass across the S_f° 0-400 range is based on measurements in 40 normal men, aged 47-66 (17).

Further heterogeneity within IDL has been suggested by recent studies in which VLDL and IDL subfractions have been subjected to in vitro lipolysis by incubation with bovine milk lipoprotein lipase (22,23). These studies, as well as parallel studies of in vivo catabolism of these fractions in the rat (24,25), have revealed multiple precursor-product relationships among the S_f° 0-400 lipoproteins. Fractions containing predominantly VLDL-I and VLDL-II yield small amounts of LDL-like products, whereas fractions containing particles in the VLDL-III/IDL-I spectrum yield products with size, density, and composition typical of LDL-II. In some subjects, however, variable amounts of lipoproteins of IDL-I size persist following catabolism of these fractions (22-24). While these particles have not yet been fully characterized, we have found in preliminary studies that following incubation with VLDL and the d>1.21 g/ml plasma fraction, they become triglyceride-enriched and metabolic precursors of LDL-II (Krauss RM, Giotas C, unpublished data). We have thus hypothesized that particles in the VLDL-III/IDL-I spectrum are capable of existing in triglyceride-rich and cholesterol-rich forms, and that these forms are interconvertible by exchange of core lipids and possibly other components (Figure 4). Preliminary evidence suggests that IDL-II also exist in cholesterol and triglyceride-rich forms. The triglyceride-rich form appears to be a metabolic precursor of LDL-IIIA, whereas the cholesterol-rich form appears to represent the larger, more buoyant portion of the LDL-I spectrum (25). Triglyceride-enriched IDL-I and IDL-II are shown as the shaded areas in Figure 3.

FIGURE 4

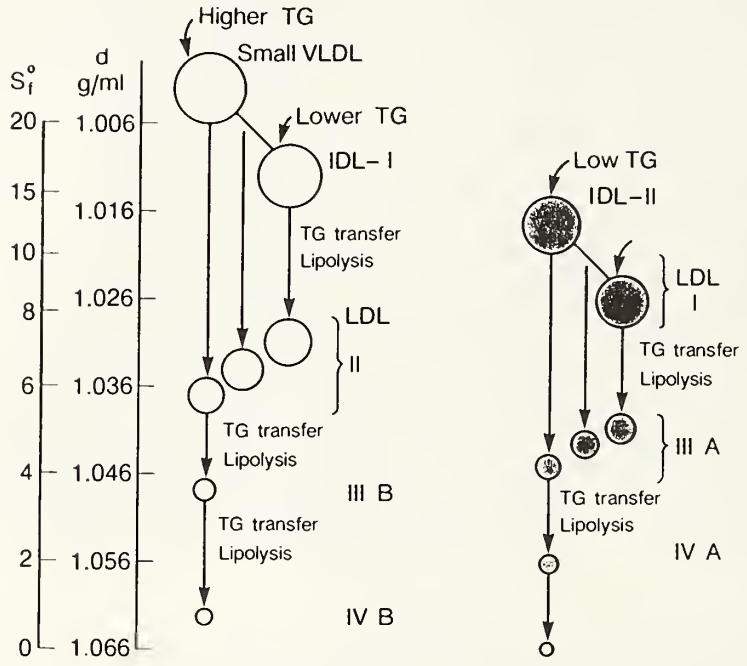
Core Lipid Substitution in IDL



The metabolic relationships described above lead to the hypothesis that at least two separate metabolic pathways give rise to LDL subpopulations: VLDL-III/IDL-I ---> LDL-II, and IDL-II ----> LDL-IIIA. These pathways are shown schematically in Figure 5. Varying input, lipid flux, and clearance of particles in these pathways could influence the distribution of the major IDL and LDL subspecies in normal and pathologic states. This scheme also summarizes preliminary observations from our laboratory that triglyceride enrichment of LDL-II and LDL-IIIA yields particles which are lipolytic precursors of the smaller and more dense LDL subspecies that are characteristically found in patients with moderate to severe hypertriglyceridemia (Krauss RM, Giotas C, unpublished data).

FIGURE 5

Dual Pathways for Production of LDL Subclasses



Similar metabolic studies using lipoprotein subfractions from patients with dysbetalipoproteinemia have suggested that β-VLDL include lipid- and apoE-loaded forms of the cholesterol-rich subspecies usually found in the IDL density range in normal subjects (23-25). Evidence recently has been reviewed that these cholesterol-enriched particles, which bear similarities to those found in cholesterol-fed animals, may have preferential involvement in the development of atherosclerosis (16).

Acknowledgments

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PHYSIOLOGIC OBSERVATIONS ON PAUCIDISPERSE LDL PARTICLE SIZE

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LDL exists as a physical continuum extending over the density range 1.006 - 1.063 gm/ml. In most individuals the major portion of LDL particles localize within a narrow region of the LDL density range and most of the lipoproteins are of similar molecular size. Such LDL we now refer to as paucidisperse (formerly monodisperse (1)). Figure 1 shows a flotation pattern of whole plasma lipoproteins at density 1.20 gm/ml. Paucidisperse LDL is shown on the left. By contrast, in another group of individuals the dispersion of LDL is widely heterogeneous with respect to density and particle size, and this LDL is designated as multidisperse. The middle and right samples are multidisperse.

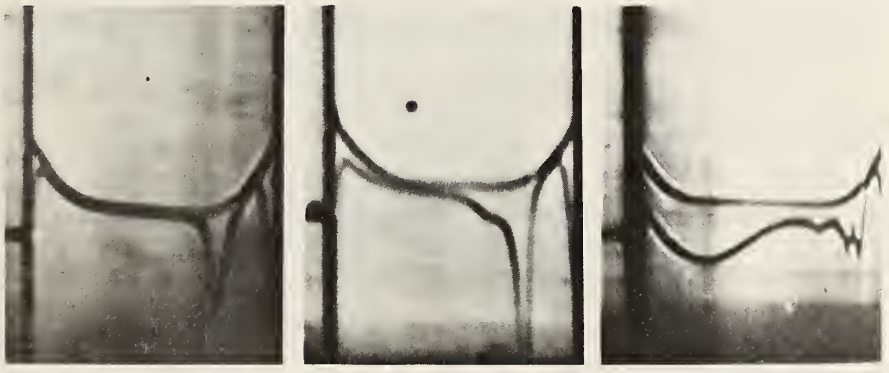

Figure 1. Dispersion of LDL. A: Paucidisperse LDL. B and C: Moderate and marked multidisperse LDL preparation. Schlieren patterns during analytical ultracentrifugation of total plasma lipoproteins in a KBr solvent (density 1.20 gm/ml). C shows a large, fast floating VLDL followed by a 3 component LDL.

The majority of healthy individuals have paucidisperse LDL. If one measures the molecular weight of such LDL on repeated occasions, one finds a wide range of molecular weights, as shown in Table 1, but the size of LDL for a given individual remains very nearly constant.

Table 1.

PHYSICAL MEASUREMENTS ON LDL

Subjects and Date of LDL Prepn	$S_{25\ 1.20}$	Hydrated Density (g/cm^3)	Mol. Wt. ($\times 10^6$ Dalton)
A (03/69)	-45	1.033	3.5
(03/70)	-46	1.032	3.5
B (03/70)	-44	1.036	3.4
(06/71)	-44	1.032	3.3
C (07/68)	-42	1.031	3.1
(10/70)	-41	1.033	3.0
D (05/58)	-42	1.030	3.0
(05/70)	-42	1.032	3.1
E (06/68)	-41	1.034	3.0
(05/70)	-42	1.034	3.0
F (05/68)	-39	1.035	2.9
(07/69)	-39	1.034	2.7
(06/70)	-40	1.032	2.9
(11/70)	-39	1.035	2.9
G (11/70)	-39	1.036	2.8
(06/70)	-39	1.034	2.9
H (10/68)	-38	1.036	2.7
(11/70)	-38	1.036	2.7
I (09/68)	-39	1.035	2.9
(11/68)	-34	1.036	2.4
(03/70)	-34	1.040	2.5

Data from reference (2).

The molecular mass of LDL has been measured in 77 caucasians, Figure 2, and extends over the range of 2 - 4 million daltons with the majority of individuals falling near the center of the range with a mean molecular weight of 3.01×10^6 daltons.

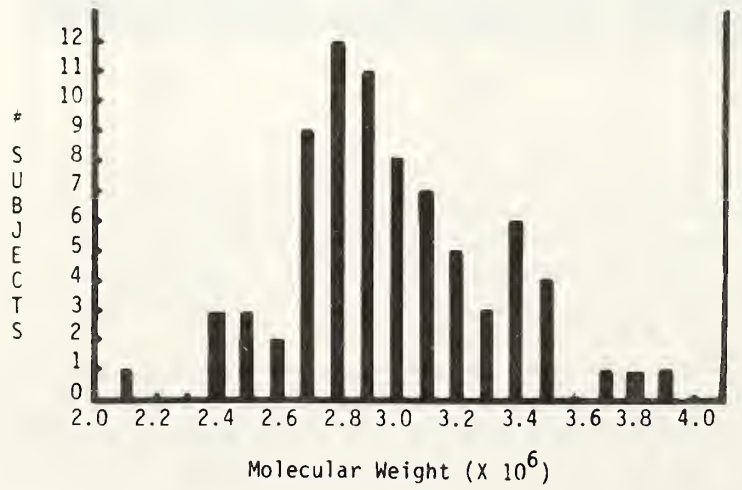

Figure 2. Molecular weight distribution of paucidisperse LDL in 77 nonhypertriglyceridemic caucasian subjects. Mean molecular weight 2.98×10^6 daltons (standard deviation 0.34×10^6 from reference (1)).

One finds a similar distribution of mass of paucidisperse LDL in 46 blacks as shown in Figure 3.

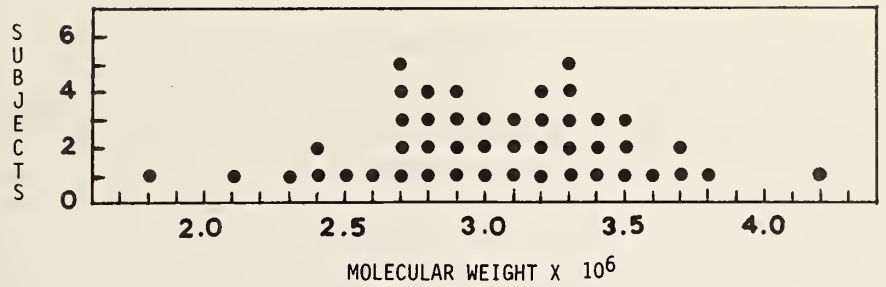

Figure 3. Molecular weight distribution of paucidisperse LDL in 46 black subjects. Mean LDL molecular weight for women (n = 26) was 2.80 x 10^6 and for men (n = 20) was 2.95 x 10^6 daltons. (Data by Wayne D. Rodriquez, unpublished).

How tightly does the body control the mass of LDL? Several observations give some insight. Figure 4, the diurnal variation of LDL size was examined in three individuals whose LDL fell in the mid-range of molecular mass. The individuals each received the same diet except for the cholesterol content which differed from 200 to 400 to 800 mg/day. Fasting LDL were isolated at 8 am on two mornings, and three samples were also analyzed after meals as indicated. Within the range of experimental error these LDL varied little in mass throughout the day.

DIURNAL CHANGE IN LDL MOLECULAR WEIGHT

A 24 Hr Study in 3 Subjects

Protocol: Diet 2400 kcal, 120 g fat
Cholesterol: Subject 1, 200 mg
Subject 2, 400 mg
Subject 3, 800 mg

Schedule:

	Meals	Blood Sampling
Day 1		8:00 am
	8:30 am	11:30 am
	11:45 am	
	4:45 pm	4:45 pm
		8:45 pm
Day 2		8:00 am

RESULTS

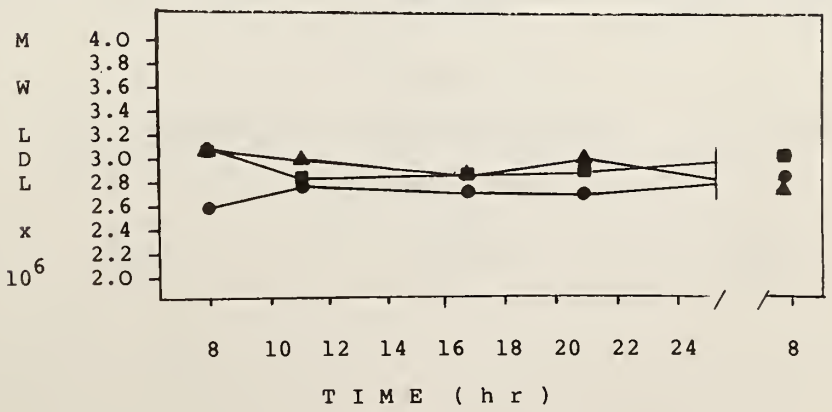

Figure 4. Diurnal variation in molecular weight of paucidisperse LDL.

The effect of diet was examined in 3 subjects over two, one-week periods, Table 2. During the first week 700 mg of cholesterol and a high saturated fat diet was administered; in the second week cholesterol intake dropped to 122 mg and the P/S ratio was reversed. LDL was isolated from fasting blood drawn at the end of each week. As seen in the left-hand column, the cholesterol content of the LDL dropped, and on the right, the molecular mass of the LDL also decreased. Though significant, the approximate 10% fall in mass was a minimal change considering the major dietary perturbation.

Table 2.

RESPONSE OF LDL MOLECULAR WEIGHT TO DIET

A 2 week study with 3 subjects

Diet: Isocaloric, constant composition except,
1st week: 700 mg cholesterol, P/S ratio 0.23
2nd week: 122 mg cholesterol, P/S ratio 2.8

Fasting blood drawn at the end of each week and LDL isolated.

RESULTS

Subjects	Cholesterol Content of LDL (mg/mg protein)	Mol. Wt. of LDL ($\times 10^6$ Dalton)
1	-0.30	-0.22
2	-0.14	-0.35
3	-0.14	-0.27
Mean	-0.30	-0.28

Some years ago, Alan Sniderman demonstrated changes in the composition of LDL when sampled across the splanchnic bed (2). Table 3, in three subjects we compared LDL isolated from the distal aorta and from the hepatic vein. The change in cholesterol and triglyceride content of the LDL were measured as was the change in particle mass. There is virtually no change in the mass of LDL as it traverses the liver.

Table 3.

CHANGE IN LDL MOLECULAR WEIGHT ACROSS THE SPLANCHNIC BED

Protocol: Blood was sampled from the distal aorta and the hepatic vein in 3 subjects. LDL was isolated.

RESULTS

Subjects	Δ LDL M.W. ($\times 10^6$ Dalton)	Δ Cholesterol Content of LDL (mg/mg protein)	Δ Triglyceride Content of LDL (mg/mg protein)
		(Aorta - Hepatic Vein)	
1	0.07	0.19	0.02
2	0.17	0.13	0.01
3	-0.05	0.04	-0.01

The physical properties of LDL can be altered in vitro, and dietary or pharmacologic perturbations will modify these in vivo. Among individuals with paucidisperse LDL there are marked differences in the molecular weight distribution of LDL particles. Yet, these individuals maintain surprisingly tight control over the metabolism of their LDL so that there is relatively little change in its unique molecular size as contrasted to other such individuals. In striking difference individuals with multidisperse LDL readily shift the physical dispersion to higher or lower sized particles (1). The explanation for these phenomena remain unknown.

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Immunochemical Methods of Isolating and Characterizing ApoB-Containing Lipoproteins

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Introduction

Recent developments in the field of plasma lipoproteins have provided further evidence for the validity of a concept which utilizes the apolipoprotein composition rather than the physicochemical properties as the main criterion for defining and classifying plasma lipoproteins [1]. The biochemical characterization of genetic disorders of lipid transport [2,3] and studies on the metabolic interactions of plasma lipoproteins [4-11] have demonstrated that apolipoproteins play an essential role not only in the formation and structural stability of lipoproteins but also in their functional capacity and specificity.

While recognizing the heterogeneity of plasma lipoproteins with respect to hydrated density, size and electrical charge, the operational classification systems cannot account for the well-documented apolipoprotein heterogeneity of ultracentrifugally, chromatographically or electrophoretically isolated lipoprotein classes [12-16]. The occurrence of apolipoproteins in non-equimolar ratios and the immunochemical patterns of non-identity or partial identity between at least some apolipoproteins have provided crucial evidence that major lipoprotein density classes consist of several distinct lipoprotein particles rather than single homogeneous lipid-protein complexes [13,17]. Although the wide distribution of apolipoproteins throughout the density spectrum disclosed a new dimension in the heterogeneity of plasma lipoproteins, it has also become evident that apolipoproteins by virtue of their chemical and immunological properties may be utilized as markers for the identification and differentiation of lipoprotein particles. These unique characteristics of plasma apolipoproteins have been used as a basis of a chemical definition and classification of plasma lipoproteins [1,12]. In its modified form [17] this concept views plasma lipoproteins as a mixture of discrete lipoprotein particles, each of which is characterized by a specific apolipoprotein composition. Lipoprotein particles which contain a single apolipoprotein are called primary or simple lipoproteins and those which contain two or more apolipoproteins are called secondary or complex lipoproteins. Each of the simple and complex lipoproteins represents a polydisperse system of particles heterogeneous with respect to the hydrated density, size and lipid/protein ratios, but homogeneous with respect to the qualitative composition of their protein moieties. Thus, individual particles of lipoprotein B (LP-B) may vary in hydrated densities [18] and lipid/protein

ratios [19] but they all contain ApoB as the sole protein component of their protein moiety. The proposed nomenclature of lipoprotein particles is based on the ABC-nomenclature of apolipoproteins: lipoprotein particles are named according to their constituent apolipoproteins [20]. For example, simple lipoproteins which only contain ApoB are called LP-B (lipoprotein B), while complex lipoproteins which have apolipoproteins B, C and E are named LP-B:C:E (lipoprotein B:C:E).

Studies on the quantitative determination [21] and distribution [14] of apolipoproteins have shown that ApoB and ApoA (A-I + A-II) form two major groups of plasma lipoproteins. The already available evidence indicates that each of these lipoprotein groups consists of both simple and complex lipoprotein particles [12,13,18,22-25]. The purpose of this report is to center on the ApoB-containing group of lipoprotein particles and to describe and discuss procedures for their isolation and separation. Preliminary results have indicated that this lipoprotein group consists of four major lipoproteins including the simple LP-B and complex LP-B:E, LP-B:C-I:C-II:C-III and LP-B:C-I:C-II:C-III:E particles [26,27].

I. Immunoprecipitation

1. Isolation of the total ApoB-containing group of lipoproteins by polyclonal antibodies to ApoB

2. Sequential isolation of distinct ApoB-containing lipoprotein particles by polyclonal antibodies to apolipoprotein E, C-III, C-II and C-I

II. Immunoaffinity Chromatography

1. Isolation of the total ApoB-containing group of lipoproteins by immunosorbers with either polyclonal antibodies to ApoB or a monoclonal antibody which binds equally to all ApoB-containing lipoproteins ("Pan B" monoclonal antibody)

2. Sequential isolation of distinct ApoB-containing lipoprotein particles by immunosorbers with either polyclonal and monoclonal antibodies to apolipoproteins E, C-III, C-II and C-I

3. Isolation of distinct ApoB-containing lipoprotein particles by specific monoclonal antibodies to ApoB

Figure 1. Immunochemical procedures for the isolation of ApoB-containing lipoproteins.

The ApoB-containing lipoproteins can be isolated by several non-immunological procedures including ultracentrifugation [28], preparative electrophoresis on solid supports [16,29], gel permeation chromatography [14,30], precipitation with anionic polyelectrolytes and bivalent cations, polyphosphates, tetracyclines, detergents and nonionic polymers [31], and affinity chromatography on concanavalin A [32]. While ApoB-containing lipoproteins may be almost completely separated from ApoA-containing lipoproteins by some of these procedures, they all lack the specificity required for differentiation of various lipoprotein forms of ApoB. This leaves the highly specific immunological methodology as the most promising means for selective isolation of distinct lipoprotein particles. The isolation and separation of ApoB-containing particles can be achieved either by immunoprecipitation or immunoaffinity chromatography (Figure 1).

The immunoprecipitation of all ApoB-containing lipoproteins and the sequential immunoprecipitation of distinct lipoprotein particles containing ApoB and other apolipoproteins require the use of polyclonal antibodies. Immunosorbers used for the same purpose can be constructed with either polyclonal antibodies or with monoclonal antibodies if they bind equally to all lipoprotein forms of ApoB and/or other associated apolipoproteins. In addition, immunosorbers can be constructed with monoclonal antibodies which recognize subspecies of ApoB-containing lipoproteins [33,34].

Methods

Study Subjects. Normolipidemic men and women, 30-60 years of age, consisted of the employees of the Oklahoma Medical Research Foundation. They were classified as normolipidemics according to the criteria recommended by the Lipid Research Clinics of the National Institutes of Health, Bethesda, Maryland [35]. All subjects were healthy, asymptomatic Caucasians with no history of familial hyperlipoproteinemia or diabetes. Patients with primary hyperlipoproteinemias were selected from those attending the Lipid Research Clinic of the Oklahoma Medical Research Foundation. None of the patients were on lipid-lowering drugs or diets expected to affect plasma lipids at least four weeks prior to blood collection. All blood donors provided informed consent. Blood samples were obtained from subjects after an overnight fast of 12 hours. Blood was drawn into tubes that contained EDTA, and the plasma samples were collected by low-speed centrifugation.

Isolation of Lipoprotein Density Classes. The very low density (VLDL, $d < 1.006$ g/ml), and two subfractions of low density (LDL_1, d 1.006-1.019 g/ml; LDL_2, d 1.019-1.063 g/ml) lipoproteins were isolated by sequential ultracentrifugation as previously described [36].

Preparation of Antigens and Antibodies. Apolipoproteins and goat polyclonal antibodies to apolipoproteins B, C-I, C-II, C-III and E were prepared and characterized as previously described [36]. The isolation and characterization of the "Pan B" monoclonal antibody and a monoclonal antibody to ApoB that specifically recognizes LP-B:E particles was carried out as previously described [37].

Immunoprecipitation. The immunoprecipitation of ApoB-containing lipoproteins was carried out according to a recent modification [27] of a previously

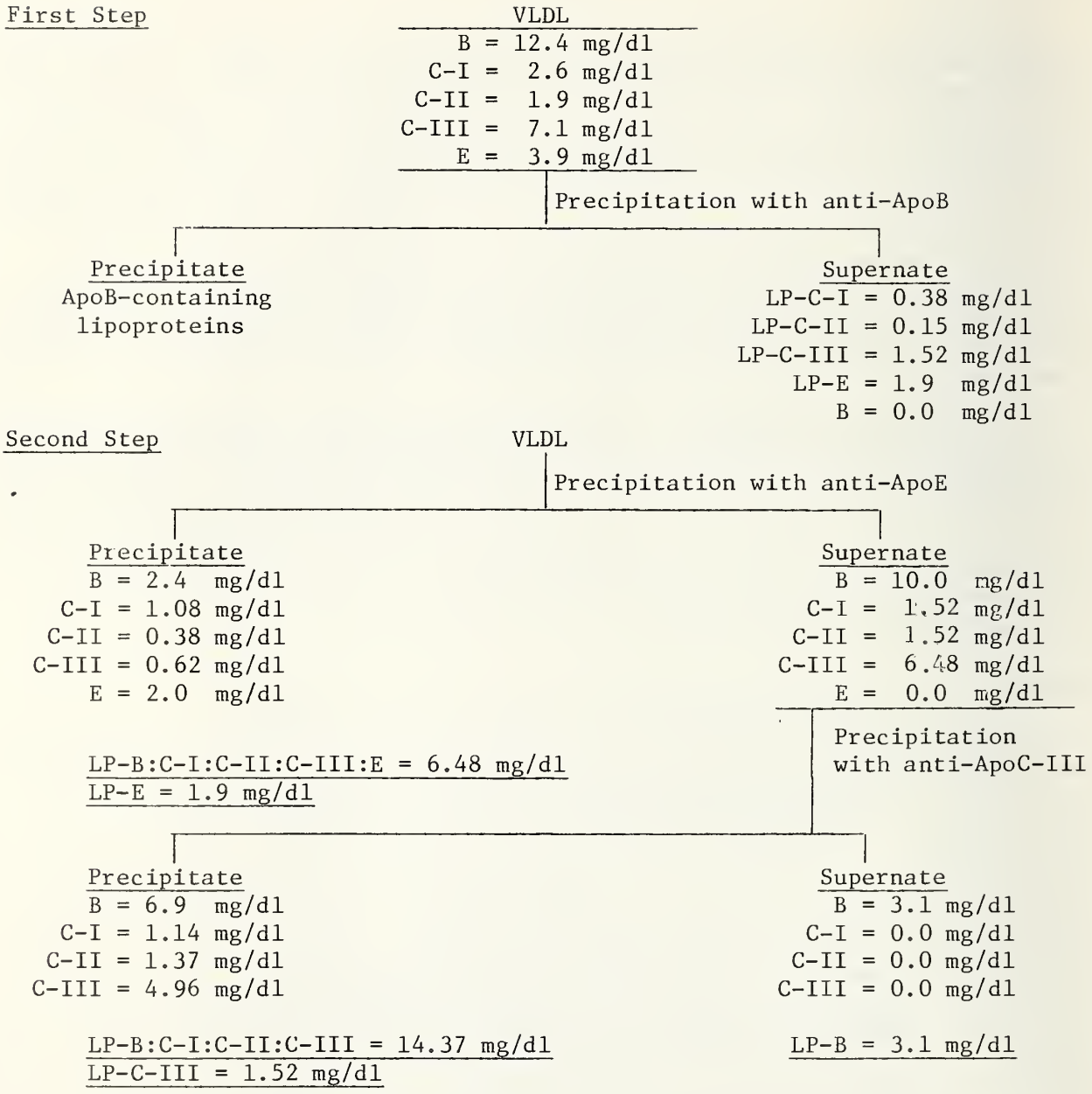

Figure 2. Scheme for the fractionation of simple and complex lipoprotein particles. Concentrations of lipoprotein particles are expressed in terms of apolipoproteins (mg/dl).

described procedure [18]. The fractionation procedure consists of two steps. The purpose of the first step is to precipitate all ApoB-containing lipoproteins from whole plasma or any other operationally-defined lipoprotein fraction in order to determine the weight proportions of apolipoproteins C-I, C-II, C-III and E bound to ApoB. An aliquot of lipoprotein preparations such as VLDL, LDL_1 or LDL_2 is mixed with a polyclonal antiserum to ApoB (IgG fraction) and the mixture is incubated for 2 h at 4°C. After low-speed ultracentrifugation

for 10 min, the supernatant fraction is removed and tested for the presence of ApoB by electroimmunoassay. If ApoB is still detectable, the precipitation is repeated until the supernate is found to be free of ApoB. The amounts of apolipoproteins C-I, C-II, C-III and E left in the supernate after complete removal of ApoB are determined by electroimmunoassays and the differences between apolipoproteins in the starting material and supernatant fraction are taken as amounts of ApoC-peptides and ApoE bound to ApoB. The second step is designed as a means of separating distinct ApoB-containing lipoprotein particles by a sequential use of polyclonal antisera to apolipoproteins to E, C-III, C-I and C-II as shown schematically in Figure 2. The order and experimental details of sequential immunoprecipitation were recently described [27]. The amounts of ApoC-peptides and ApoB coprecipitated with a polyclonal antiserum to ApoE are estimated as differences between the levels of these apolipoproteins in starting lipoprotein preparation and the supernatant fraction remaining after the removal of precipitated lipoproteins. The amounts of apolipoproteins C-I, C-II and B coprecipitated with antibodies to ApoC-III were calculated in the same manner. After the completion of sequential immunoprecipitation with antisera to apolipoproteins E, C-III, C-I and C-II, the supernatant fraction only contains LP-B particles. Fractionation procedure may be monitored by double diffusion analysis and polyacrylamide gel electrophoresis. Concentrations of simple and complex lipoproteins are expressed in terms of their apolipoproteins (mg/dl).

Immunoaffinity Chromatography. Polyclonal and monoclonal antibodies to ApoB and other apolipoproteins were purified by ammonium sulfate precipitation [38] and coupled to the cross-linked agarose activated with N-hydroxysuccinamide (Affi-gel 10, Bio-Rad Laboratories, Richmond, CA) as previously described [38]. A comparison of various dissociating agents showed that 3 M sodium thiocyanate is the most effective desorbent for lipoproteins bound to agarose-based immunosorbers [39]. To minimize the exposure time between the lipoprotein and dissociating agent, the columns for the immunoaffinity chromatography were first packed with 25 ml of Sephadex G-25 followed by 10 ml of antibody-coupled Affi-gel and another protective layer of 5 ml Sephadex G-25 [38,39]. The Sephadex G-25 layers were three times bed volumes of the antibody-coupled gel and served as a desalting column for immediate separation of lipoproteins from the dissociating agent. The chromatography and determination of immunosorber capacity were performed as previously described [39]. The binding capacity of an immunosorber was defined as the amount of apolipoprotein (in mg) retained per milliliter of immunosorber [39].

Analytical Methods. Triglycerides, cholesterol and cholesterol esters were quantified by gas-liquid chromatography as described by Kuksis et al. [40]. The quantitative determination of apolipoproteins B, C-I, C-II, C-III and E was carried out by electroimmunoassays developed in this laboratory [21].

Results

Sequential Immunoprecipitation of Discrete ApoB-Containing Lipoprotein Particles. The sequential immunoprecipitation was applied to the fractionation of discrete ApoB-containing lipoprotein particles in VLDL, LDL_1 and LDL_2 from normolipidemic subjects and patients with various types of dyslipoproteinemia. As shown in Figure 2, the complete precipitation of ApoB-containing

lipoproteins in the first step of the procedure causes a marked, but not complete, coprecipitation of ApoC-peptides and ApoE. Very small amounts of simple lipoprotein particles LP-C-I (0.1-0.5 mg/dl), LP-C-II (0.1-0.3 mg/dl), LP-C-III (0.5-1.0 mg/dl) and LP-E (0.2-0.5 mg/dl) are present in the supernatant fractions of all three low density lipoprotein classes isolated from normolipidemic subjects. However, in hypertriglyceridemic patients, the levels of LP-C-III and LP-E are 2-3-fold higher than in normals. Immunochemical evidence suggests that these minor non-ApoB-containing lipoproteins occur as separate particles [27]. Chromatography of the same lipoprotein density classes on concanavalin A showed results similar to those of immunoprecipitation including the recovery of small amounts of LP-C-I, LP-C-II, LP-C-III and LP-E in the unretained fractions.

The second step of the sequential immunoprecipitation (Figure 2) is initiated by complete precipitation of ApoE-containing lipoproteins with polyclonal antibodies to ApoE. The precipitated lipoproteins consist of LP-E and LP-B:C-I:C-II:C-III:E (abbreviated LP-B:C:E); in some individual donors this fraction also contains LP-B:E particles in VLDL, LDL_1 and, particularly, LDL_2. The amount of LP-E is estimated by measuring the ApoE content in the supernatant fraction remaining after the complete precipitation of ApoB-containing lipoprotein in the first step of this procedure (in the example shown in Figure 2, the amount of ApoE in LP-E was estimated to be 1.9 mg/dl and that of LP-B:C:E 2.0 mg/dl). The occurrence of LP-B:C:E particles was verified by the isolation of an almost identical complex lipoprotein with immunosorber containing a monoclonal antibody to ApoE. The supernatant fraction remaining after precipitation of ApoE-containing lipoproteins was treated with polyclonal antibodies to ApoC-III and the ApoC-III-free supernate was removed for further fractionation. The precipitated lipoproteins consist of LP-C-III and LP-B:C-I:C-II:C-III (abbreviated LP-B:C). The concentration of LP-C-III is determined in the same manner as already described for LP-E. To verify the apolipoprotein composition of LP-B:C, the antigen-antibody complex was dissociated under acidic conditions [18] and the released ApoC-peptides were identified by polyacrylamide-gel electrophoresis and electroimmunoassay. The supernatant fraction remaining after the precipitation with antibodies to ApoC-III is treated sequentially with antisera to ApoC-I and ApoC-II to remove small amounts of these apolipoprotein-containing lipoproteins. The final remaining supernate only contains LP-B particles. Thus, the results of sequential immunoprecipitation of lipoprotein particles in VLDL, LDL_1 and LDL_2 of both normolipidemic and hyperlipoproteinemic subjects showed that ApoB occurs mainly in three lipoprotein forms including the simple lipoprotein LP-B and complex lipoproteins LP-B:C and LP-B:C:E (Table 1). The LDL_2 fraction of some normolipidemic and hyperlipoproteinemic patients, particularly those with familial hypercholesterolemia, contained LP-B:E rather than LP-B:C:E particles.

It is well-established that LP-B particles are the major lipoprotein form of ApoB in LDL_2 [18,19,27,32,39,41-44]. Results of this study show that LP-B particles are also present in VLDL. As shown in Table 1, in VLDL of normolipidemic subjects, LP-B accounts on an average for 10% and LP-B:C and LP-B:C:E for 90% of ApoB-containing lipoproteins. However, in LDL_1 and LDL_2, LP-B particles account for 55% and 75% of the total amount of ApoB-containing lipoproteins, respectively. The LDL_2 fraction contains even in normolipidemic subjects a relatively high percentage of complex ApoB lipoprotein particles. In one-half of the examined subjects, LDL_2 contained LP-B:E particles.

Table 1. Concentrations of major simple and complex lipoproteins in density classes of normolipidemic and hyperlipidemic subjects.

Lipoprotein Particles	VLDL	LDL_1	LDL_2
		mg/dl	
Familial Hypercholesterolemic Heterozygotes (n = 3)			
LP-B	2.6 ± 4.2*	8.9 ± 2.4	160 ± 24
LP-B:C:E**	3.6 ± 1.6	4.1 ± 1.9	35 ± 16
LP-B:C	1.8 ± 1.2	2.0 ± 1.8	1 ± 0.7
Primary Hypertriglyceridemic Phenotype IV (n = 5)			
LP-B	0.2 ± 0.4	4.1 ± 3.6	33 ± 16
LP-B:C:E	29 ± 11	4.1 ± 1.9	13.4 ± 10.1
LP-B:C	44 ± 27	5.6 ± 6.1	7.4 ± 6.6
Normals (n = 8)			
LP-B	0.55 ± 1.0	3.4 ± 3.0	49 ± 18
LP-B:C:E***	3.4 ± 2.3	2.1 ± 1.3	11.2 ± 6.5
LP-B:C	1.4 ± 1.4	0.7 ± 0.4	6.0 ± 4.8

* Mean ± S.D.
** Values shown represent LP-B:C:E particles in VLDL and LDL_1; in LDL_2 they represent LP-B:E particles
*** In the LDL_2 subclass, four subjects had LP-B:C:E particles (13.5 mg/dl) and four subjects had LP-B:E particles (9.5 mg/dl)

The apolipoprotein composition of LP-B:C and LP-B:C:E particles isolated from VLDL, LDL_1 and LDL_2 is characterized by an increasing percentage of ApoB and decreasing percentages of ApoC-peptides and ApoE with increasing density. The analyses of neutral lipids showed very characteristic lipid profiles of LP-B and LP-B:C:E particles. The LP-B particles from all three density classes had cholesterol esters as the major neutral lipid constituent (60% in VLDL; 59% in LDL_1; and 75% in LDL_2); with increasing density, the percentage of triglyceride decreased and that of free cholesterol increased. On the other hand, the LP-B:C:E particles were characterized by triglyceride as the main neutral lipid constituent (73% in VLDL; 67% in LDL_1; and 60% in LDL_2); free and esterified cholesterol increased slightly with increasing density of particles. The neutral lipid composition of LP-B:C particles was also characterized by triglyceride as the main neutral lipid (78% in VLDL), but the percentages

of triglyceride in LP-B:C from LDL_1 and LDL_2 were lower than those of corresponding LP-B:C:E particles. The LP-B:E isolated from LDL_2 of normolipidemic subjects had a higher percentage of cholesterol esters and a lower percentage of triglyceride than the corresponding LP-B:C:E particles. The presence of these characteristic ApoB-containing lipoproteins in all segments of the low density spectrum (S_f 0-400) indicates that each of these lipoprotein forms is a polydisperse system of particles characterized by changing proportions of individual apolipoproteins and lipids. The increasing percentage of LP-B and decreasing percentages of LP-B:C and LP-B:C:E from VLDL though LDL_2 result from the lipolytic processes in which LP-B:C:E and LP-B:C are identified as substrates and LP-B as the product of this catabolic reaction [27].

In patients with endogenous hypertriglyceridemia and familial hypercholesterolemia (Table 1), significant changes in the levels and distributions of major ApoB-containing lipoprotein particles reflect alterations in the production, degradation and uptake of these lipoproteins. All three density classes from patients with the heterozygous form of familial hypercholesterolemia had significantly higher levels of LP-B particles which, in the LDL_2 fraction, were 4-5-fold higher than in normals or in patients with hypertriglyceridemia (Table 1). In VLDL and LDL_1, there was no significant difference between normals and hypercholesterolemics in the levels of LP-B:C and LP-B:C:E particles. However, in LDL_2, hypercholesterolemic patients had consistently higher concentrations of LP-B:E particles than normals. This profile of lipoprotein particles in hypercholesterolemia is compatible with altered uptake [9,10] of LP-B and LP-B:E particles, although increased synthesis of these two types of lipoproteins may also be a contributing factor [45].

All major lipoprotein forms of ApoB were also found in patients with endogeneous hypertriglyceridemia (Table 1), but their concentrations and distributions differed markedly from those of normolipidemic and hypercholesterolemic subjects. The lipoprotein profile was characterized by highly elevated levels of triglyceride-rich LP-B:C and LP-B:C:E in VLDL and low levels of LP-B in LDL_2. In LDL_1 and LDL_2, the concentrations of LP-B:C and LP-B:C:E were only slightly increased in comparison with those of normal subjects. The accumulation of complex lipoprotein particles of very low hydrated densities reflects the overproduction and, most probably, decreased lipolytic degradation of these two distinct triglyceride-rich lipoproteins [45]. Ongoing studing on the profiling of ApoB-containing lipoprotein particles in other dyslipoproteinemic states reflect to some extent the presented profiles of normolipidemic, hypercholesterolemic and hypertriglyceridemic subjects depending on the nature of any particular biochemical defect of lipid transport.

The apolipoprotein and lipid composition of ApoB-containing lipoproteins from patients with dyslipoproteinemia shows similarity with that of normolipidemic subjects, but some quantitative differences cannot be ruled out. This also applies to concentrations and composition of minor ApoB-containing lipoprotein particles such as LP-B:C-I, LP-B:C-II, LP-B:C-I:E, etc. Further studies are needed to define such differences and assess their metabolic significance. It should also be pointed out, that within each characteristic profile of lipoprotein particles, there are variations between individual subjects in the concentrations of simple and complex lipoproteins. It remains to be established to what extent these quantitative differences correlate with underlying defects of lipid transport.

Isolation of ApoB-Containing Lipoproteins by Immunoaffinity Chromatography. Although the sequential immunoprecipitation is a useful procedure for identification and quantification of discrete ApoB-containing lipoproteins, immunoaffinity chromatography is a more suitable method for their isolation. This applies particularly to lipoprotein particles bound to their corresponding antibodies. While it is rather difficult and cumbersome to recover antigenic components from precipitated antigen-antibody complexes, it is relatively simple to elute antigens retained on immunosorbers. Since the first successful application of immunoaffinity chromatography to the isolation of ApoB-containing lipoproteins by Kostner and Holasek [46], this highly specific isolation method has already been extensively exploited by many investigators for the isolation of both apolipoproteins and lipoproteins. The list of these contributions is too long to be adequately cited and presented in this report. Instead, this presentation will only address application of immunoaffinity chromatography to the isolation of ApoB-containing lipoproteins based on the most recent studies conducted in this laboratory.

Immunoaffinity chromatography employing polyclonal or monoclonal antibodies to ApoB and other apolipoproteins associated with ApoB can be used for the isolation of all ApoB-containing lipoproteins or, similarly to sequential immunoprecipitation, for the sequential isolation of discrete ApoB-containing lipoproteins. A systematic study [39] of the optimal conditions for constructing and operating immunosorbers with polyclonal antibodies to ApoB has shown that immunosorbers with the highest capacity were obtained by cyanogen bromide activation of Sepharose and that among various dissociating agents tested, 3 M sodium thiocyanate was found to be the most effective desorbent for bound lipoproteins. To minimize the time of contact between the lipoprotein particles and dissociating agent, it was found advantageous to construct immunosorbers with a protective layer of Sephadex G-25 placed below the immunosorber portion. In this manner, the Sephadex G-25 serves as a desalting column by separating the lipoproteins from the dissociating agent. By allowing lipoproteins only a brief contact with sodium thiocyanate, the protective layer of Sephadex G-25 minimizes the potentially disruptive effect of this agent on lipoprotein structure. Two other aspects of immunosorber methodology are concerned with the specificity of immunosorbers and the possibility of non-specific adsorption of lipoproteins and proteins other than those corresponding to the attached antibodies. The monospecificity of antibodies is tested by double-diffusion analyses, crossed immunoelectrophoresis and electroimmunoassay carried out with pure apolipoproteins and plasma. We have found albumin to be a suitable protein for testing the non-specific binding of immunosorbers because of its capacity to bind fatty acids and lysolecithin. The results of such experiments showed an almost complete recovery of albumin in unretained fractions (97-100%). Thus, the non-specific binding of albumin is minimal; the retained fractions are also free of plasma globulins when tested by double diffusion analyses. The recovery of apolipoproteins or lipoproteins is between 80-98%.

Immunoaffinity chromatography of whole plasma from normolipidemic subjects on anti-ApoB immunosorbers showed that ApoC-peptides and ApoE were retained together with ApoB indicating that these former apolipoproteins are components of the complex ApoB-containing lipoprotein particles identified as LP-B:C and LP-B:C:E. Comparison of these retained lipoprotein particles with the parent whole plasma by crossed immunoelectrophoresis showed no change in their

mobilities. Repeated chromatography of retained fractions over the same immunosorber yielded no detectable apolipoproteins in the unretained fractions suggesting that the elution of lipoproteins by sodium thiocyanate caused no dissociation of apolipoproteins from the retained ApoB-containing lipoproteins. Similarly, the lipid composition of retained fractions remained unchanged after repeated chromatographic runs.

Comparison of parent VLDL and LDL_2 preparations with VLDL and LDL_2 retained on anti-ApoB immunosorbers showed very little difference in the apolipoprotein and lipid composition. The immunodiffusion patterns and flotation rates of parent VLDL and LDL_2 were very similar to those of the corresponding retained fractions. Similar results were obtained with VLDL from hypertriglyceridemic patients. Electron microscopy of lipoproteins from whole plasma retained and eluted with sodium thiocyanate from the anti-ApoB immunosorber revealed a population of spherical particles which did not differ from those in the original plasma. All these findings show that, under optimal experimental conditions, the ApoB-containing lipoproteins retained by immunosorbers with polyclonal antibodies to ApoB have chemical, physical and immunological properties similar, if not identical, to those of their corresponding parent density classes.

A systematic study on the sequential isolation of discrete ApoB-containing lipoprotein particles, analogous to the already described sequential immunoprecipitation, has not yet been performed. However, serially connected immunosorbers were used for the isolation of LP-B particles from LDL_2 [39]. In this approach, LDL_2 preparations were passed sequentially over anti-ApoA-I, anti-ApoC, anti-ApoD and anti-ApoE immunosorbers. The final unretained fraction contained immunochemically homogeneous LP-B particles. The LP-B particles accounted for 70-80% of the LDL_2 from normolipidemic subjects, a range of values similar to that obtained for LP-B by sequential immunoprecipitation of LDL_2 (Table 1). The chemical and physical characteristics of LP-B particles were consistent with LDL_2-like particles. Furthermore, LP-B particles were recognized by high-affinity binding sites on cultured fibroblasts and regulated the activity of HMG-CoA reductase like the parent LDL_2. These results show that LP-B particles isolated by sequential use of immunosorbers retain their structural and biological integrity.

All ApoB-containing lipoprotein particles can also be isolated by immunosorbers constructed with monoclonal antibodies if they bind equally to all lipoprotein forms of ApoB ("Pan B" antibody). We have recently described [37] the preparation of a monoclonal antibody with comparable binding affinities to soluble ApoB, chylomicrons, VLDL and LDL. When coupled to agarose, this antibody allowed complete removal of ApoB-containing lipoproteins from plasma of normolipidemic, hypercholesterolemic and hypertriglyceridemic subjects. It appears that this antibody binds to a rather stable domain of ApoB that is not altered by the interaction with lipids and other apolipoproteins. The "Pan B" monoclonal antibody was used for the isolation of ApoB-containing lipoproteins from whole plasma, VLDL and LDL_2 [38]. The chemical composition and morphological properties of retained fractions were virtually identical to those of parent density classes and the corresponding retained fractions isolated by immunoaffinity chromatography with polyclonal antibodies to ApoB. The capacity of immunosorber with affinity-purified "Pan B" monoclonal antibody was 3-fold higher than that of an immunosorber with affinity-purified polyclonal antibodies. Recovery of ApoB and reproducibility were also somewhat better compared to polyclonal antibodies.

Another, probably most interesting application of monoclonal antibodies is to the isolation of some subspecies of ApoB-containing particles by the use of specific monoclonal antibodies to ApoB. Using immunoaffinity chromatography with a selected monoclonal antibody to ApoB, we were able to isolate a subpopulation of low density lipoprotein particles from a normolipidemic plasma in a single step procedure [33]. These particles were homogeneous in terms of size (20 nm), flotation coefficient (S_f 9.5) and electrophoretic mobility (beta). The protein moiety consisted of apolipoproteins E and B in a molar ratio close to 2. The lipid composition was characterized by 47% cholesterol, 5% triglycerides and 48% phospholipids. The most likely explanation for these results is that the selected monoclonal antibody recognizes a particular epitope on ApoB which is fully and most favorably expressed in the described LP-B:E particles. Other types of ApoB-containing particles, with the corresponding epitope altered or hidden by lipids, are bound with an insufficient affinity to be retained or they pass through the column completely ignored by the antibody. The net result of retention of the described LP-B:E particles and their separation from other ApoB-containing particles.

Conclusions

1. Apolipoprotein B occurs in the form of several distinct lipoprotein particles of very low and low hydrated densities.

2. The major lipoprotein particles of VLDL and LDL are the simple lipoprotein LP-B, and complex lipoproteins LP-B:C:E, LP-B:E and LP-B:C. Minor lipoprotein particles include simple lipoproteins LP-C-I, LP-C-II, LP-C-III and LP-E, and complex lipoproteins LP-B:C-I, LP-B:C-II, etc.

3. Discrete lipoprotein particles can be identified and separated by sequential immunoprecipitation or immunoaffinity chromatography and measured by quantification of apolipoproteins.

4. All simple and complex ApoB-containing lipoproteins constitute polydisperse macromolecular systems heterogeneous with respect to hydrated density and lipid and apolipoprotein composition.

5. Major ApoB-containing lipoproteins have specific apolipoprotein and lipid composition. In all density regions, the LP-B particles are characterized by cholesterol esters and the LP-B:C:E, LP-B:E and LP-B:C by triglycerides as the major lipid constituent. In complex lipoproteins, the content of ApoB increases and the contents of ApoC-peptides and ApoE decrease with increasing densities of particles.

6. Dyslipoproteinemias are characterized by distinct profiles of ApoB-containing lipoproteins. Hypercholesterolemic states are characterized by elevated concentrations of cholesterol ester-rich LP-B particles and the hypertriglyceridemic states by increased levels of triglyceride-rich LP-B:C and LP-B:C:E.

7. Immunoaffinity chromatography is a more suitable method than immunoprecipitation for the isolation of discrete ApoB-containing lipoprotein particles. Isolated lipoprotein particles maintain their structural and biological integrity.

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Overview of Apolipoprotein B Structure and Metabolism

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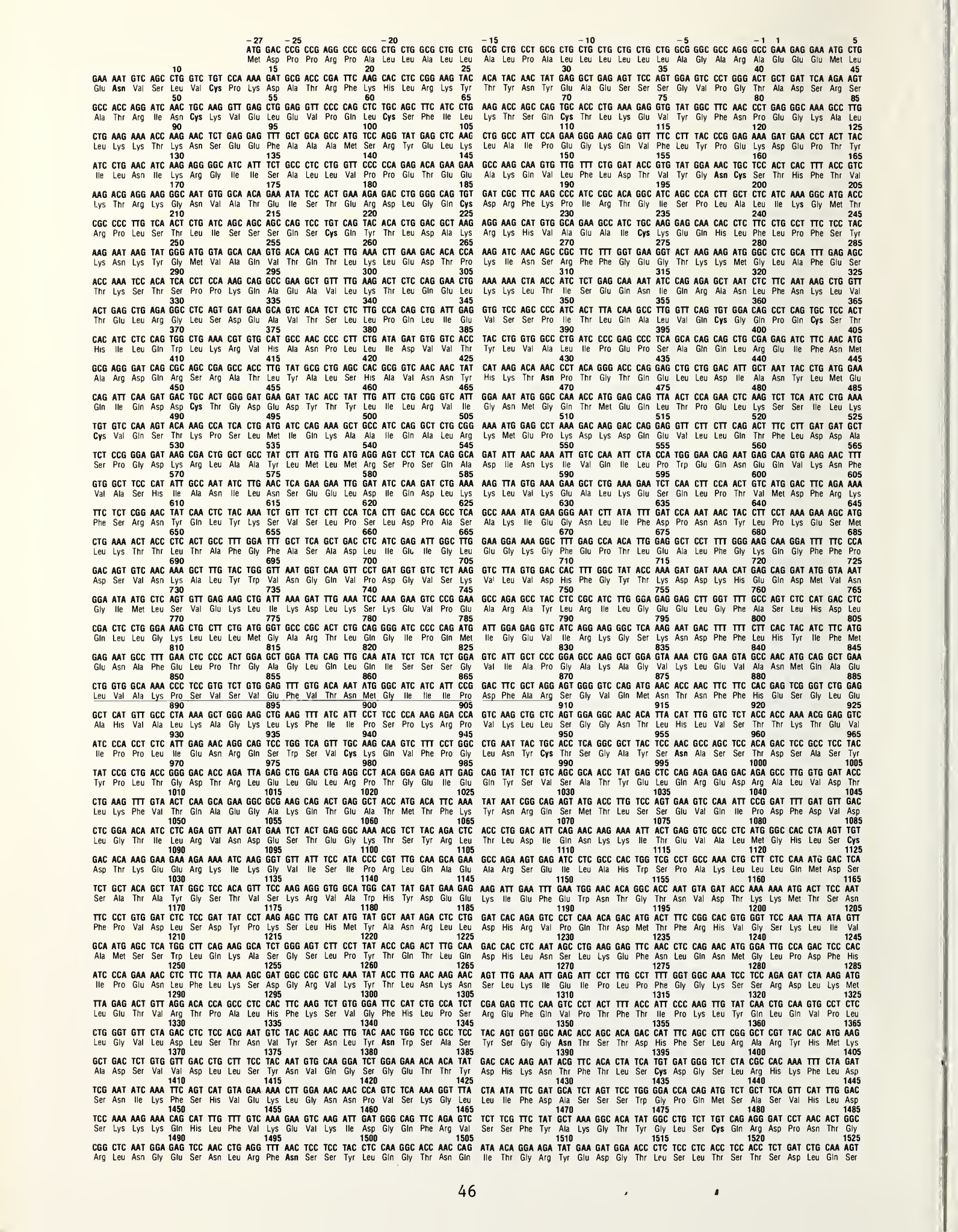
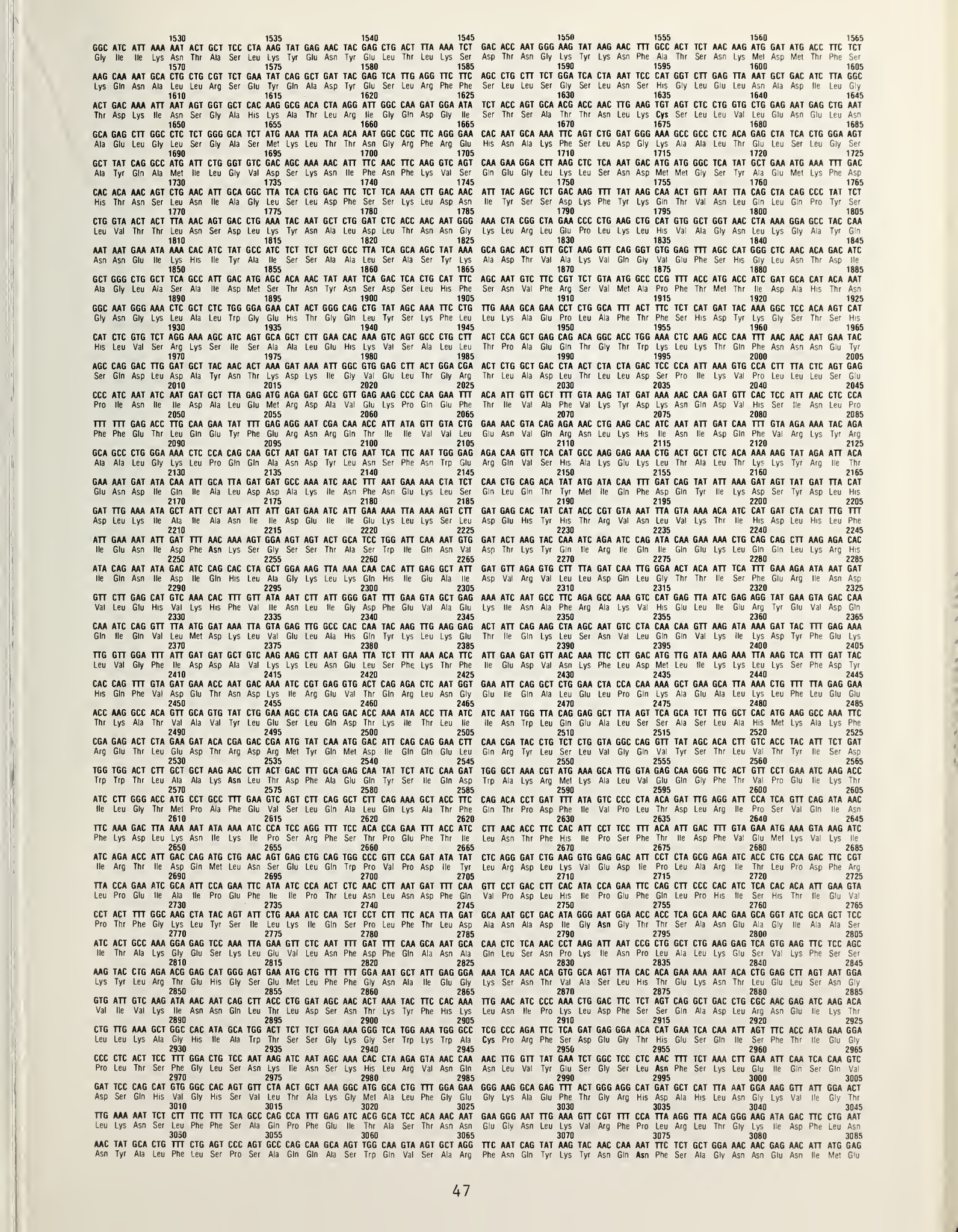
Introduction

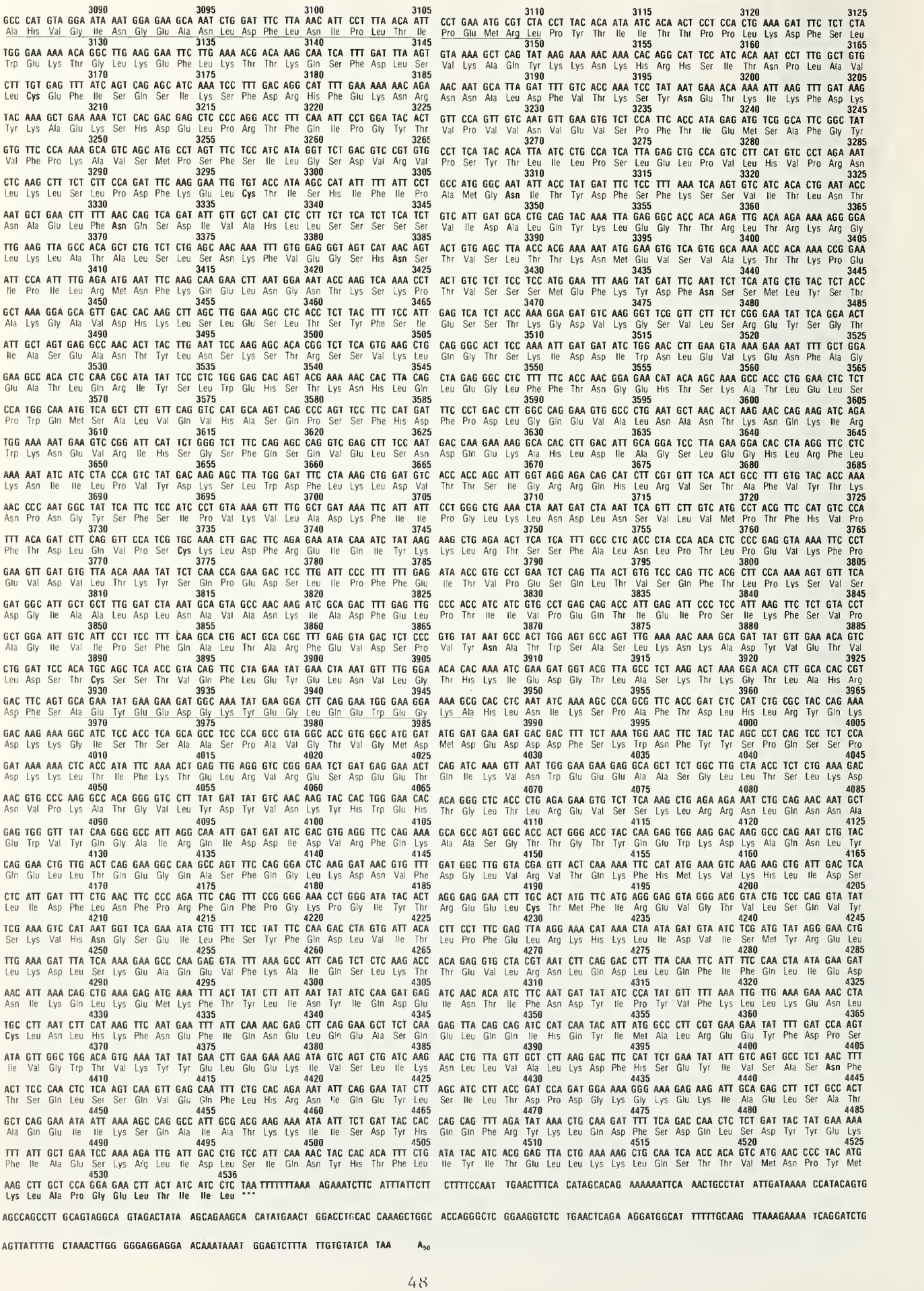
Human apolipoprotein (apo) B is a major apolipoprotein on chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL) (1-4). ApoB has been one of the most interesting as well as the most frustrating apolipoproteins studied in the last decade due to its insolubility in aqueous solution, and aggregation in buffers containing $NaDodSO_4$, urea, or guanidine HCL. A significant insight into the molecular heterogeneity of the B apolipoprotein was the observation that in the rat (5) and human (4) apoB exists in two forms which are separable by $NaDodSO_4$ gel electrophoresis. In man the two forms are designated apoB-48 and apoB-100. The molecular weight of apoB-100 has been controversial, and values ranging from 8-400 kDa have been reported (1-4,6,7). The heterogeneity in the molecular weight of apoB-100 has been attributed to both the propensity of apoB-100 to aggregate, and to be cleaved by proteolytic enzymes (3,4,8). The origin of apoB-48 has also been of great interest. ApoB-48 has been considered to be the product of a apoB mRNA, or to result from the proteolytic cleavage of apoB-100 (4,9,10).

Structural Analysis of ApoB-100

A number of the questions pertaining to apoB have been resolved following the cloning and sequence analysis of the entire cDNA of apoB-100 (11-13). The cDNA and derived amino acid sequences of human apoB-100 determined in our laboratory are as follows:







AGCCAGCCTT GCAGTAGGCA GTAGACTATA AGCAGAAGCA CATATGAACT GGACCTGCAC CAAAGCTGGC ACCAGGGCTC GGAAGGTCTC TGAACTCAGA AGGATGGCAT TTTTTGCAAG TTAAAGAAAA TCAGGATCTG

AGTTATTTTG CTAAACTTGG GGGAGGAGGA ACAAATAAAT GGAGTCTTTA TTGTGTATCA TAA A_{50}

Figure 1. Complete cDNA and derived amino acid sequence of human apoB-100.

The mRNA of apoB-100 is 14.1 kb, and the apolipoprotein contains 4536 amino acids. Fifteen of the twenty five cysteine residues are present in the first 500 amino acids. This unusual distribution of cysteine residues suggests there is a high degree of ordered structure in the amino terminal end of apoB-100. There are 25 potential N-linked glycosylation sites in apoB-100, the majority of which are located in the middle portion of the apolipoprotein. Of particular interest is the recent finding that apoB-100 is acylated with fatty acids (14). The presence of covalently attached fatty acids to apoB-100 will have a dramatic effect on the hydrophobic properties of the apolipoprotein, and would be anticipated to have a significant effect on the lipid binding properties of the apolipoprotein and its association with the lipoprotein particle.

An analysis of the primary amino acid sequence of apoB-100 revealed no extensive linear sequences of hydrophobic residues, and there are no long stretches of amphipathic helixes. Thus the interaction of apoB-100 with LDL involves both secondary and tertiary structure, and the apolipoprotein would be anticipated to have a complex conformation on the lipoprotein particle. Evidence to support the importance as well as the complexity of the conformation of apoB-100 on plasma lipoproteins was obtained by analysis of VLDL and LDL with apoB-100 monoclonal antibodies (15). The exposure of epitopes on apoB-100 was different on VLDL and LDL, and antibodies which are directed toward the LDL receptor binding domain(s) reacted primarily with LDL. These results were interpreted as indicating that the conformation of apoB-100 changes during the sequential lipolysis of VLDL to IDL, and finally to LDL (15).

ApoB Biosynthesis

The structural relationship of human apoB-100 and apoB-48 has not been definitively established. In man apoB-48 and apoB-100 have been proposed to be synthesized by the intestine and liver, respectively (4). The availability of cDNA probes to apoB-100 has now provided the opportunity to evaluate the species of apoB mRNA present in human liver and intestine. In the human liver a single 14.1 kb mRNA was observed by Northern blot analysis (16-18). Cultured normal human hepatocyctes and HepG-2 cells secrete into the media only apoB-100 (19). These results established that human liver contains a single 14.1 kb apoB-100 mRNA and only apoB-100 is synthesized and secreted from the hepatocyte.

Evaluation of the species of apoB secreted by the human intestine has been more difficult due to the problems of obtaining samples for culture and analysis. Studies in our laboratory revealed that the human intestine contains a 14.1 kb mRNA identical to the apoB-100 mRNA present in the liver (16-18). In addition a second 7.5 kb apoB mRNA is present in the intestine of sufficient size to code for apoB-48 (Fig 2).

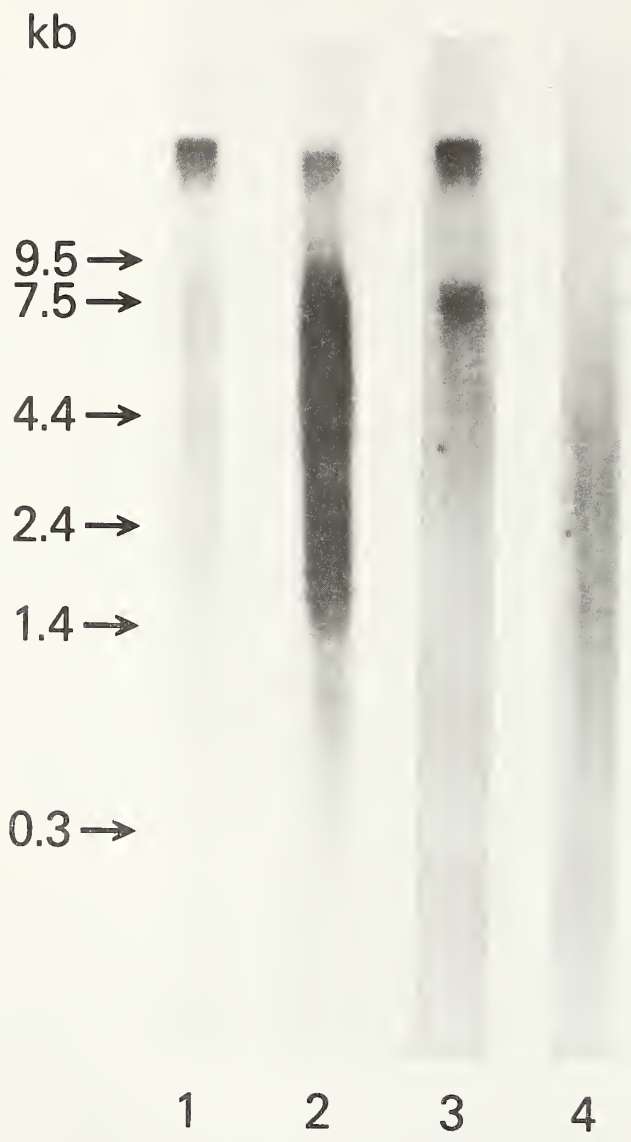


Figure 2. Autoradiogram of the Northern blot filter hybridization of human liver and intestine mRNA utilizing a 5' cDNA probe of apoB-100 mRNA. Lanes (1) liver poly(A)$^+$ RNA; (2) intestinal total RNA; (3) intestinal poly(A)$^+$ RNA. (4) intestinal poly(A)$^-$ RNA.

Analysis of the 7.5 kb apoB mRNA with cDNA probes directed toward the 5', middle, and 3' end of the apoB-100 mRNA revealed that the 7.5 kb mRNA is the 5' half of the apoB-100 mRNA. Thus the 7.5 kb mRNA would code for an apolipoprotein which would be the amino terminal half of apoB-100. These results are consistent with the immunological studies which indicated that apoB-48 is derived from the amino terminal half of apoB-100 (15,20). Detailed studies on the form(s) of apoB secreted from cultured human enterocytes have not been reported. The major question of whether the 7.5kb mRNA is functional and codes for apoB-48 remains to be answered. Additional studies will be required to establish if apoB-48 is derived by proteolytic cleavage from apoB-100 or is the product of a separate apoB-48 mRNA.

Modulation of the expression of the apoB-100 gene is of particular interest, however, studies in this area are only just being initiated. Initial studies in our laboratory have been directed to establish if LDL can modulate the biosynthesis and secretion of hepatic apoB-100. Incubation of HepG-2 cells with LDL resulted in a down regulation of HMG-CoA reductase, and a decrease in the cellular level of apoB-100 mRNA as well as a reduction in the secretion of apoB-100 (21). These studies have suggested that there is coordinate regulation of the LDL receptor, HMG-CoA reductase, and apoB-100 biosynthesis and secretion.

Metabolism of Plasma Lipoproteins Containing ApoB

Based on our current information we can now propose an updated working model for the metabolism of the apoB containing plasma lipoproteins. Of major importance is the recent data reviewed above that apoB-100 containing lipoproteins may be secreted by the intestine. The metabolic pathways for apoB containing lipoproteins synthesized and secreted from the intestine and liver can be conceptualized as consisting of two lipoprotein cascades as outlined below in Figure 3

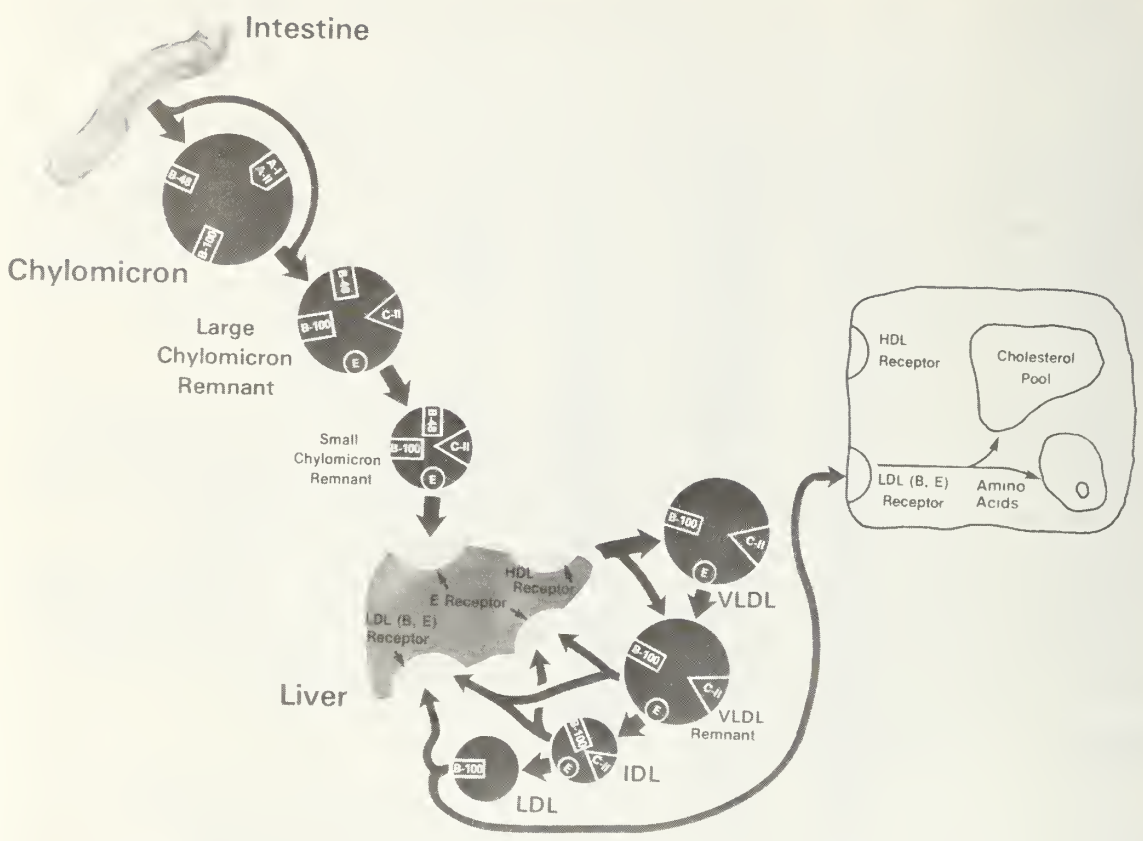


Figure 3. Overview of human lipoprotein metabolism

The first apoB cascade consists of chylomicrons containing either apoB-100 or apoB-48 secreted by the intestine (Fig. 3). Following secretion, the lipoproteins acquire the E and C-II apolipoproteins from HDL. The triglycerides on newly secreted chylomicrons undergo rapid hydrolysis by lipoprotein lipase which is activated by apoC-II (22,23). With triglyceride hydrolysis the chylomicrons are gradually converted to chylomicron remnants which are taken up by the chylomicron remnant or apoE receptor system in the liver (Fig. 3) (24).

The second apoB cascade involves triglyceride rich apoB-100 containing lipoproteins with a hydrated density of VLDL which are secreted by the liver. The triglyceride rich VLDL undergo hydrolysis by apoC-II and lipoprotein lipase as outlined above for the intestinal chylomicrons (Fig. 3). During the metabolism of VLDL some of the small VLDL remnants and IDL are removed from the circulation by the apoE or chylomicron remnant receptor, and the LDL receptor (Fig. 3). LDL is the major metabolic product of the VLDL cascade. LDL is catabolized from plasma via the LDL receptor on peripheral cells (25), and the liver (26). Interaction of LDL with its receptor initiates receptor-mediated endocytosis and catabolism of LDL (25). This apoB-100 LDL receptor pathway provides the cholesterol for cellular function including membrane synthesis in peripheral cells.

Summary

Major new insights have developed during the last several years in our knowledge of apolipoprotein structure and function as a result of the cloning and structural analysis of apoB-100. The elucidation of the structure of apoB-100 will now provide the necessary information for an analysis of the LDL receptor binding site(s) on apoB-100, the biosynthetic pathway of apoB-48, and the study of the dyslipoproteinemias characterized by an overproduction of apoB-100 (e.g. combined hyperlipidemia) or a deficiency of the B apolipoproteins including abetalipoproteinemia as well as hypobetalipoproteinemia.

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COUPLED LIPID TRANSFER AND LIPOLYSIS IN INTRAVASCULAR PROCESSING/REMODELING OF APOPROTEIN B CONTAINING LIPOPROTEINS

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Genetic and metabolic control of lipoprotein production and catabolism are important regulators of plasma levels of triglyceride-rich and cholesterol ester-rich lipoproteins. At the same time, processes that occur in the plasma compartment play major roles in lipoprotein interconversions and remodeling that not only are important in understanding lipoprotein heterogeneity, but also plasma lipid metabolism. Pertubations of lipoprotein metabolism which alter levels of triglyceride-rich as compared to cholesterol ester-rich lipoproteins, likely contribute to lipoprotein heterogeneity and remodeling by influencing pathways well recognized to occur in the human plasma compartment.

A number of processes influence lipoprotein remodeling in plasma. Separating particle core events from surface events, some processes are distinct for core vs surface, while others are shared. In the particle core, triglyceride lipolysis by either lipoprotein lipase or hepatic lipase is the major mechanism for conversion of triglyceride rich to more cholesterol ester rich remnants and LDL. Neutral lipid transfers, mediated by neutral lipid transfer protein (LTP) exchange, remove, and/or add core lipids in all lipoproteins. LCAT is important in producing cholesterol esters that contribute to core volume.

At the surface lipolysis may play a role in removal of phospholipids. Transfer of surface components occurs spontaneously through the acqueous phase, such as with free cholesterol and apoproteins, either as individual molecules or as part of molecular aggregates, such as when excess surface remnants are shed from VLDL during core triglyceride hydrolysis. Surface constituent transfer may also be enhanced by specific transfer proteins, and this may be especially important for phospholipid (1). Surface properties are also modified by LCAT in depleting the surface of free cholesterol, and producing lysolecithin.

This paper will first focus on the effects of lipolysis and neutral transfer as separate entities, and then integrate them to build the hypothesis that neutral lipid transfer processes coupled with lipolysis are important in regulating cholesterol carriage, and lipoprotein structure and heterogeneity in human plasma.

Lipolysis

Triglyceride hydrolysis is well recognized as a major pathway for catabolizing triglyceride rich lipoproteins. Removal of core triglyceride is a prerequisite for allowing chylomicrons and VLDL to move down the lipolytic cascade towards remnants, IDL, and LDL. With the knowledge that triglyceride can transfer into LDL and HDL via neutral lipid transfer processes (2), it is also important to understand how triglyceride is removed from these cholesterol ester-

rich lipoproteins.

Studying the affinities of bovine milk lipoprotein and human hepatic lipases for different lipoproteins, we found that Km's of each enzyme on isolated lipoprotein classes are not markedly different. Lipoprotein lipase has a lower Km or higher affinity, for VLDL triglyceride than hepatic lipase, but hepatic lipase has a lower Km for HDL triglyceride relative to lipoprotein lipase (3). Interestingly, the affinities of both enzymes for LDL were found to be greater than for either VLDL or HDL (3). Expressing Km's in terms of the number of separate particles in each lipoprotein class, we can define marked differences between lipoproteins. To hydrolyze the amount of triglyceride represented by the Km values, 200-300 more particles are required for HDL compared to VLDL. These calculations help to understand the need for more enzyme and/or more time to hydrolyze triglyceride in LDL, and particularly HDL, compared to VLDL. Thus, a single lipase molecule would require access to 200-300 more HDL particles compared to VLDL to achieve equivalent hydrolysis. These differences in Km, however, do not clarify whether a particular lipase will select a particular lipopotein in human plasma where a mixture of different lipoproteins is present. We addressed this question by studying mixed competitive systems containing mixtures of triglyceride-rich and cholesterol ester-rich lipoproteins, in in vitro incubations with either lipoprotein or hepatic lipase.

In mixtures of VLDL and HDL we found that at VLDL triglyceride concentrations similar to normal human plasma (100mg/dl) that both VLDL and HDL triglyceride were well hydrolyzed by lipoprotein lipase. However, reaching higher amounts of VLDL (equivalent to plasma triglyceride levels of 300mg/dl), lipase acted only on VLDL, resulting in cessation of lipolysis in HDL. A similar effect was observed in incubating VLDL with lipopotein lipase in the presence of LDL. But lipolysis in LDL was totally inhibited only at higher relative amounts of VLDL (3).

Similar experiments clearly defined a much higher affinity of hepatic lipase for HDL and LDL, relative to VLDL (3). These results suggest that in moderate hypertriglyceridemia removal of triglyceride from LDL and HDL is largely dependent on hepatic lipase. Moreover, while each lipase can hydrolyze each lipoprotein, under conditions present in plasma hydrolysis of triglyceride in a given lipoprotein by a specific enzyme will depend on the relative concentrations between lipoprotein subclasses.

In assessing the role of phospholipolysis in removing excess surface components, we compared phospholipid to triglyceride hydrolysis in the major lipoproteins with each enzyme. As compared to triglyceride hydrolysis, phospholipolysis is most efficient in VLDL and lowest in LDL. In comparing the two enzymes hepatic lipase is a more efficient phospholipase than lipoprotein lipase on all lipoproteins (4). Calculations derived from these experiments indicate that on catabolism of VLDL to LDL, that phospholipolysis can only account for 25-40% of the phospholipid that must be removed as VLDL is converted to LDL. Similarly, phospholipolysis alone, at least in vitro, will not provide sufficient phospholipid removal to allow conversion of HDL_2 to HDL_3. We propose, therefore, that shedding of unhydrolyzed phospholipid must also occur to rid particles of excess surface as core volume is reduced.

Lipolysis alone can account for major core depletion of triglyceride rich lipoproteins. i.e. particles in which the major core component is triglyceride. But for the cholesterol ester rich lipoproteins, LDL and HDL, other mechanisms

must be invoked to account for major remodeling in plasma, mechanisms which allow for substantial loss of the major core constituent, cholesterol ester. Recent work has demonstrated that neutral lipid transfer processes will allow for substantial removal of cholesterol esters from LDL and HDL, and that those neutral lipid transfers coupled with lipolysis, can explain major remodeling of lipoproteins in plasma, and do play a role in lipoprotein heterogeneity.

Coupled Neutral Lipid Transfer and Lipolysis

Cholesterol esters transfer from LDL and HDL to VLDL, with concomitant triglyceride transfer from VLDL to both LDL and HDL was first described by Nichols and Smith in 1965 (2). Later work has shown that these neutral lipid exchanges are dependent on a specific transfer protein present in plasma of some (human, rabbit, monkey) but not all (rat, pig) mammalian species (5-7). This protein variously called cholesterol ester transfer protein or neutral lipid transfer protein (LTP) has been recently purified and has a molecular weight of 72,000 Daltons (8). LTP circulates in plasma, and is likely bound to lipoprotein surfaces, especially to HDL (9).

How LTP mediates neutral lipid exchanges is not yet known. Morton and Zilversmit have shown that the transfer protein exchanges neutral lipid in relation to the relative masses of donor and acceptor particles irrespective of the nature of the core lipid (10). The protein does seem to facilitate transport of di- and monounsaturated cholesterol esters more than others (11). Studies in our laboratories have demonstrated that these transfer proteins mediate exchange processes that not only account for major shifts of neutral lipids in the plasma compartment, but when coupled with the activity of lipoprotein triglyceride lipases can regulate chemical and structural remodeling of human plasma lipoproteins (12,13). These data demonstrate that VLDL not only serves as a precursor of LDL in plasma, but the relative size of the VLDL pool to the LDL (and HDL) pool are important in determining lipoprotein modeling, and likely metabolism (14).

Using in vitro model systems, we incubated normal LDL or HDL (12,13) cholesterol ester-rich LDL or HDL (12,15) with VLDL. In the presence of either lipoprotein deficient d >1.21 g/ml plasma containing LTP, or of purified LTP, we found that up to 2/3 of LDL or HDL cholesterol ester can be transferred to the triglyceride rich particles. Concomitant with these cholesterol ester losses, LDL (or HDL) became enriched with triglycerides transferred from VLDL. These large shifts in neutral lipids were independent of LCAT activity or apoproteins, but did closely correlate with ratios of triglyceride rich to cholesterol ester-rich particles, and the time those particles were together in the same system. Conditions used for these experiments were similar to those of normal human plasma, in that the amount of LTP was not rate limiting, but that VLDL:LDL ratios and incubation times controlled the degrees of triglyceride-cholesterol ester exchange. Artificial triglyceride emulsions can fully substitute for VLDL as a triglyceride donor and cholesterol ester acceptor in exchange incubations with LDL and HDL. (13,16). Thus, none of the VLDL constitutents (e.g. apoproteins) are specifically required for transfer processes to occur. Of relevance to what may occur in vivo (see below), Tall et al, demonstrated that cholesterol ester transfers are significantly enhanced by concomitant lipolysis of triglyceride rich particles by lipase (17).

Also, important to these remodeling processes, is the fact that

triglyceride acquired by LDL (and HDL) can be hydrolyzed by triglyceride lipases (12,13). This allows for cholesterol ester again becoming the major core lipid in LDL or HDL particles, which are now smaller and relatively depleted of cholesterol esters. While cholesterol ester losses account for most of the decrease in size and molecular weight, LDL exposed to these processes also loses phospholipids and free cholesterol, but not apoprotein B. Thus, the same amount of protein remains on a particle now considerably decreased in size and lipid content, a situation which likely affects apoprotein B conformation and apoB interaction with its receptors at cell surfaces.

Another point to consider is that in losing cholesterol esters both LDL and HDL act as the major source for augmenting the cholesterol ester content of VLDL particles. The latter effect likely has important implications on VLDL metabolism as discussed below. These processes, then, allow for exchange of molecules that cannot be degraded in plasma, cholesterol esters, with molecules that can, triglyceride. In LDL and HDL, lipid transfer coupled with triglyceride hydrolysis provides a mechanism for depletion of core lipids, and is thus a major determinant of size, density, cholesterol ester content, and subpopulation distribution of the two lipoproteins.

Do these mechanisms, initially characterized in vitro, relate to what occurs in vivo? Likely, yes. Evidence that the pathways we have defined in vitro, do operate in vivo can be derived from a number of sources.

In situations where the triglyceride rich lipoprotein pool is small compared to the cholesterol ester rich lipoprotein pool, we would predict that without sufficient acceptor particles, the transfer of cholesterol esters from LDL and HDL would be limited. Familial hypercholesterolemia is an example where the LDL cholesterol ester pool is large compared to the VLDL pool. In this condition, the predicted accumulation of cholesterol ester in LDL, is confirmed by studies of LDL composition and physical properties. Hypercholesterolemic LDL contains more cholesterol ester and less triglyceride per particle than normal LDL (18,19). LDL is lighter and larger than normal with increased molecular weight relative to normals (18,19). These changes are even more pronounced in homozygous compared to heterozygous patients (20). In a situation where no apoB containing lipoproteins are present in plasma, abetalipoproteinemia, no triglyceride acceptors are present for cholesterol esters generated in HDL. In this disease, most HDL consists of an abnormally large HDL_2 subpopulation, containing more then twice the cholesterol esters than normal HDL_2 (15). In most normal subjects, HDL_3 is the major HDL subpopulation). These excess cholesterol esters can be removed from abeta HDL by incubation with normal VLDL in vitro, and after lipase hydrolysis of transferred triglyceride, abeta HDL is converted towards normal.

Data from our in vitro remodelling systems would predict that in situations where the triglyceride-rich lipoprotein pool is large compared to the LDL/HDL pool that neutral lipid transfer processes would be more active, and result in cholesterol ester depleted, triglyceride enriched LDL and HDL. Further, with hydrolysis of triglyceride transfered into LDL and HDL, these lipoproteins should be smaller and denser than in normals. Since in vitro, triglyceride hydrolysis in LDL and HDL requires more lipase than in VLDL, we would predict that not all transferred LDL or HDL triglyceride would be hydrolyzed in vivo. These findings have indeed been reported by a number of investigators. LDL and HDL in hypertriglyceridemia are smaller, denser and contain less cholesterol ester (21-25).

Studies from our laboratories have not only confirmed these observations, but have shown in fact, that the structure and compositional properties of LDL and HDL are closely and very significantly correlated to the relative ratios of triglyceride to cholesterol-rich lipoprotein pools, over ranges of plasma lipid levels from subjects with abetalipoproteinemia to severe hypertriglyceridemia (26,27). Decreases in LDL and HDL size, buoyancy, absolute cholesterol ester content and cholesterol ester to triglyceride ratios, core to surface content, and lipid to protein ratios all occur in a graded manner with increasing plasma triglyceride levels. At the same time, the triglyceride rich lipoprotein pool may carry more then half the cholesterol esters present in plasma (27). The alterations just listed can be reversed by lowering plasma triglyceride levels with lipid lowering agents in hypertriglyceridemia (27). This suggests that the changes described are related largely to conditions in the plasma compartment, and not to the basic genetic metabolic defect.

Can the lipoprotein changes observed in chronic dyslipidemic states also occur during more acute perturbations of the plasma triglyceride-rich lipoprotein pool, in vivo? Studies in collaboration with Dr. Yvon Carpentier (Free University of Brussels) analyzed plasma lipoproteins before, during, and after 6 hours intravenous infusion of long chain triglyceride emulsions in normolipemic volunteers. Interestingly, even over this relatively short time period, changes in LDL and HDL cholesterol ester and triglyceride, as a function of plasma triglyceride levels were similar to those we previously documented in chronic dyslipidemia. Moreover, with increasing triglyceridemia during the infusions, progressively more plasma cholesterol esters were carried in the triglyceride rich (d <1.019 g/ml) lipoprotein pool. At higher plasma triglyceride concentrations (> 500 mg/dl) one third of plasma cholesterol ester transferred into VLDL-IDL (Y. Carpentier, R. Deckelbaum, unpublished data). Calculations derived from these data revealed that the fluxes of cholesterol ester from LDL and HDL into the triglyceride rich pool during the in vivo infusions surpassed transfer rates in in vitro model systems (with equivalent lipemic conditions) by 1 1/2 to 2 fold.

Since, in vitro, cholesterol ester transfer to VLDL is substantially enhanced during lipolysis (17), the infusion data suggests that the very active lipolysis we documented during these infusions contributes to the rapid neutral lipid transfers we recorded in vivo. Similar rapid transfers of neutral lipids might be predicted to occur during the increased triglyceride lipolysis associated with post-prandial lipemia.

The observations outlined above provide for understanding of how the relative size of the triglyceride-rich vs cholesterol ester rich lipoprotein pools can influence lipoprotein structure, heterogeneity, and metabolism. The final product in terms of lipoprotein structure and composition will depend on the dynamics of lipid exchange determined by activity of the lipid proteins, relative ratios of triglyceride vs. cholesterol ester-rich lipoprotein pools, and lipolysis. Data from in vitro studies (13), suggests that the amount of exchange protein in human plasma will not be rate limiting in most situations, but that relative amounts of triglyceride rich vs cholesterol ester rich particles will have a strong influence on remodeling. A scheme depicting how neutral lipid transfers and lipolysis can model plasma lipoproteins is presented in Figure 1.

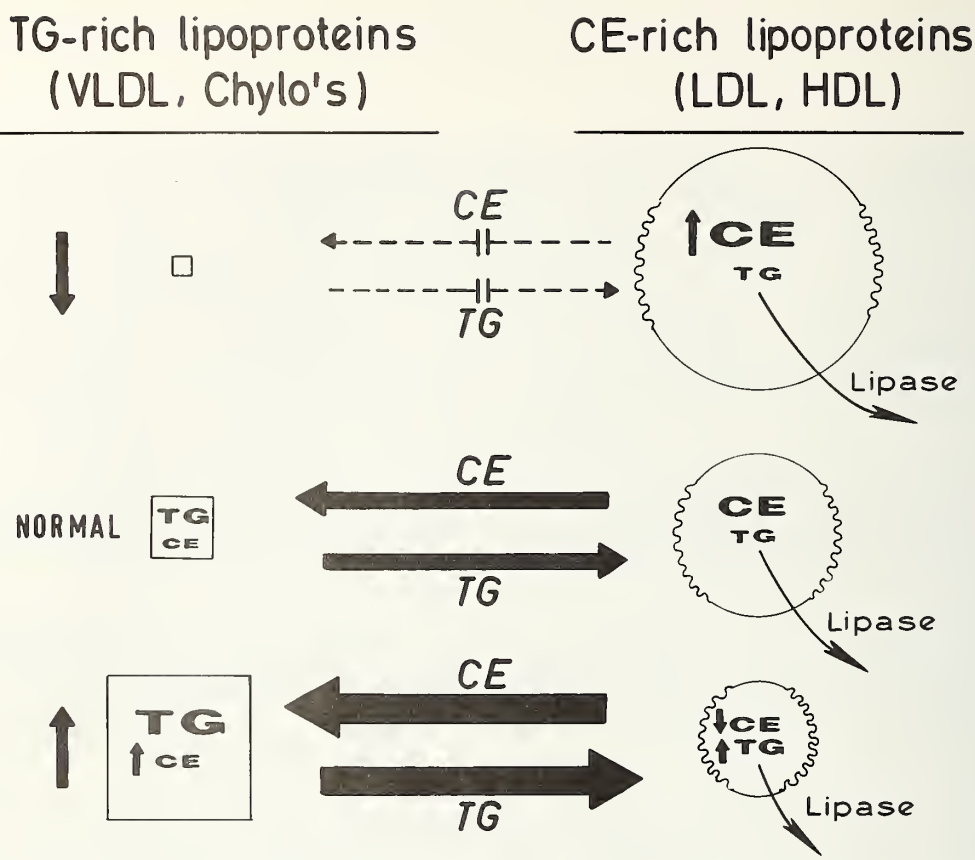

Figure 1. The roles of neutral lipid exchange processes and lipolysis in remodeling human plasma lipoproteins. The scheme depicts 3 situations where relative sizes of the triglyceride-rich lipoprotein pool (left) and that of the cholesterol ester-rich lipoproteins (right) vary. Under normal conditions (MIDDLE PANEL), cholesterol esters which accumulate in LDL or HDL transfer to the triglyceride-rich lipoprotein pool, with concomitant transfer of triglycerides to LDL or HDL. The LDL and HDL triglycerides are hydrolyzed by lipases resulting in LDL and HDL composition and structure as defined in normal subjects. In the TOP PANEL the triglyceride-rich lipoprotein pool is almost absent or very small compared to that of the cholesterol ester-rich lipoproteins. Such situations exist in abetalipoproteinemia or severe familial hypercholesterolemia. Under these conditions, there is no or relatively little ability to transfer cholesterol esters out of HDL or LDL, producing abnormally large particles enriched in cholesterol esters. In the BOTTOM PANEL the triglyceride-rich lipoprotein pool is large relative to that of the cholesterol ester-rich lipoproteins, e.g., familial hypertriglyceridemia. With a large triglyceride-rich pool for accepting cholesterol esters and transferring triglycerides, neutral lipid exchange increases, and LDL and HDL are depleted of cholesterol esters and acquire triglycerides. The efficiency of triglyceride lipolysis by lipase (hepatic lipase, lipoprotein lipase or other lipases) determines the amount of triglyceride remaining in LDL or HDL but under most conditions, most but not all, acquired triglyceride is hydrolyzed in LDL and HDL. Therefore, the size of LDL and HDL decreases as they are depleted of cholesterol ester, and acquired triglyceride. Because not all acquired triglyceride is hydrolyzed, its contribution to LDL and HDL increases. At the same time, the triglyceride-rich lipoprotein pool carries more cholesterol with particles enriched in cholesterol esters.

Because changes in triglyceride-rich particles may vary according to particle number, plasma residence time or other factors (see text), the triglyceride rich lipoproteins are grouped as pools and depicted by square boxes. Effects of neutral lipid exchange and lipolysis remodeling are depicted for LDL and HDL as the effects on individual particles. The figure also suggests that apoprotein conformation on LDL or HDL may be substantially altered by changing particle size and composition. (Reproduced from reference 14).

As already discussed the scheme provides a plausible explanation of large sized, cholesterol ester rich LDL's and HDL, in situations with low (or absent) levels of triglyceride-rich lipoproteins. At the opposite extreme it provides a mechanism for small, cholesterol ester depleted LDL and HDL in hypertriglyceridemia. Of interest are the possibilities for change of triglyceride-rich lipoproteins in hypertriglyceridemia. In hypertriglyceridemia, it is expected that increased cholesterol esters in VLDL (acquired by transfer) will have an effect on particle metabolism. If, for example, cholesterol ester enrichment an VLDL is associated with a slowly turning over VLDL pool, the individual VLDL particles could contain too many cholesterol esters to reach LDL via lipolysis, and they would then be cleared by other pathways. With an equivantly large but rapidly turning over VLDL pool although total cholesterol esters carried in VLDL might be the same, an individual VLDL particle with a short plasma residence time would not accumulate excess cholesterol ester, and could then convert to LDL. We have previously reported that neutral lipid exchange processes act on IDL (12), thus allowing influx and efflux of cholesterol esters from this particle population as well. We propose, then, that these exchange processes will also influence whether catabolic products of triglyceride-rich particles will be cleared from the plasma compartment before or after conversion to LDL.

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SESSION I. DISCUSSION ON ISOLATION, ORIGIN, AND PROCESSING OF ApoB-CONTAINING LIPOPROTEINS

DR. FISHER: Dr. Brewer, in 1964, Sam Gurin and I described the fatty acylation of ApoB, and asked at that time about the effects that fatty acylation might have on the hydrophobic properties of the protein and our inability to work with this protein. Now Dr. Hoeg is raising questions about translational modification and the effects that this has on the metabolism of ApoB. What is the extent of fatty acylation of the ApoB that you find intracellularly in the A-beta patients?

DR. BREWER: In the A-beta patients, the "classic" ones, that we've seen the protein in, we haven't as yet had sufficient protein from those patients to determine whether ApoB is acylated or not. One of the obvious questions for the post-translational processing of Apo-B is the role that acylation may play and also the same is true for A1 and E since they are also both acylated. Is the acylation playing a role in taking the protein through a particular circuitous route through the cell and therefore is very important for biosynthesis and secretion? The other post-translational modification that might be very important is carbohydrate addition. Defects in the regulation of this group of disorders in which at least two major post-translational modifications, acylation and carbohydrate addition are present may relate to some of the defects in the different patients.

The role of metabolism is obviously a critical one. It is possible that one of the properties of ApoB that makes it so difficult to work with is the degree of acylation that it has in the plasma. The degree of acylation of ApoB may be very important in terms of its ability to bind to the same particle and not to move around versus all of the other apoproteins. Obviously, the size may also contribute to that.

At this point acylation is an interesting observation but we don't know yet its full physiological significance.

DR. GINSBERG: Dr. Brewer the studies of LDL's down-regulation of ApoB message and your contrasting studies with LDL deficiency states reminds me of a study that we've just carried out on a patient with cholesteryl ester storage disease which might be considered in terms of how the cytoplasmic cholesterol pool sees LDL deficiency. We found markedly increased ApoB production rates, probably in VLDL, but certainly kinetically in LDL. We for various reasons treated the patient with mevinolin and showed suppression of ApoB production and secretion of ApoB-containing lipoproteins into plasma. Do you have any data concerning the effect of mevinolin on ApoB message?

DR. BREWER: It decreases.

SPEAKER: Dr. Brewer, why were you not able to suppress the ApoB message in the HepG2 cells forever? Why did you lose this suppression?

DR. BREWER: We don't know yet. Perhaps that relates to some adaptation by the cell. We wondered why it doesn't go down and change or whether there is a certain period of time that cholesterol is the ligand on LDL. We would like to assume that there is coordinated control between the biosynthesis, ApoB production, and secretion of particles. But we don't know that cholesterol is the ligand. Maybe there is only a certain timeframe in which cholesterol is able to carry out its metabolic function and then it is modified or oxidized. But we have not enough data on the time course yet to know.

DR. WITZTUM: Dr. Alaupovic, we've been isolating lipoproteins for many years and we all know that certain apoprotein will "spin-off", and be lost. In essence, what we are saying is certain components of a particle that we assume were originally there, whereas others won't. So there is some kind of physical force that is used to separate the particles, to separate what was originally there. Now we are going to immunochemical techniques and we are saying that an antibody that has an affinity for a component on a particle is going to separate and precipitate out that particle. How do we know that in a similar manner that when we separate a particle by an antibody that we are not just separating these new particles because the affinity of the antibody is so much greater for ApoB than for ApoE and how would you assess that?

DR. ALAUPOVIC: The only way you can assess that is by comparing the different types of antibodies used for the same purpose. In order words, if you can repeatedly show reductions of various types of antibodies, namely those that may have higher or more affinity for a certain particle, you show that you always isolate the same or very similar type of lipoprotein particle. I think that we are not modifying particles by use of immunological procedures.

DR. WITZTUM: But isn't that like saying that if you do an ultracentrifugation with a very constant density gradient and every time you separate an LDL you always get the same exact LDL density subfraction, that the procedure is justified? You could always say you are only spinning off the ApoE.

DR. ALAUPOVIC: Then let's compare immunological procedures with non-immunological procedures. This can be done in some cases by the use of Con-A. We are trying now to develop a system of checks and balances whereby the immunochemical procedure can be justified as a legitimate procedure. But at this point I see no other alternative than the use of various types of reasonably well-defined antibodies on the one hand and immunological procedures like Con-A as another.

DR. KRAUSS: Dr. Alaupovic, with regards to the last observation that you described where there is an single monoclonal antibody, presumably with a single epitope, that pulls out a subpopulation that contains both B and E, you didn't describe what the possible epitope recognition site on the ApoB might be. Do you have any information on that?

DR. ALAUPOVIC: We don't know. It is the work of an associate, Dr. Cory. His feeling is that a particular epitope must be present. He doesn't know what particular region this is, but it is an epitope that is probably conformationally in such a form that it picks up specifically the ApoB or the ApoB epitope. It is difficult to fully explain this, but it seems to me that it depends very much on the conformation that is imposed upon B by the presence of E.

DR. WEISGRABER: Dr. Brewer, this morning you presented some results from experiments in which you used c-DNA probes from the five prime end and showed that they reacted with B-48 message, whereas a probe in the three prime end didn't react with the message. Recently, you published data indicating that an antibody made to a synthetic peptide from the carboxy terminal region of B-100 reacted with B-48. Could you comment on this apparent contradiction?

DR. BREWER: I don't think it's a contradiction because we don't really know the specificity for the antibody studies, whether they're conformational or not. It's the age-old question of how we are going to interpret the antibody binding studies in which we are inhibiting the receptor binding domain. We don't know in those particular studies whether there is a similar conformational epitope, whether it is a

is a linear epitope and what role the area of the pocket might have in terms of steric hindrance of the antibody.

From the standpoint of looking at the B-48 versus B-100 in terms of the probes it is pretty straightforward that all of the probes that have been utilized that are in the carboxy terminal region of the protein, that are in the three prime mRNA don't react with the B-48 "mRNA". That is, the mRNA that is 7.5 kilobases. The important aspect of that mRNA is to make sure that mRNA can translate a protein, which is one of the things we are working on. So, there are limitations of the poly-adenylation of mRNA and, secondly, whether or not that message is in fact translatable.

DR. CHAIT: Dr. Brewer, have you had the opportunity to add mevalonate to your HepG2 cells and look at the effect on the RNA levels?

DR. BREWER: Yes, we're doing that right now.

DR. HAVEL: This relates to Dr. Weisgrabers' question. John Kane has approached the question of whether B-48 is just the N-terminal half of B-100 in two ways. One is by using a series of bacterial endoproteinases and looking at the arrays of polypeptides which are produced thereby and the observation is consistent only with the B in the internal half. That is because all of the polypeptides in each of the endoproteinases are identically arrayed with B-48 and some of the proteins of B-100 except for one overlapped species which is present only in B-100. So that is inconsistent with there being downstream representation. The other is by constructing about eight or nine focal polyclonals that are site-specific antibodies to synthetic peptides represented by the N-terminal one-half of B-100 and they all react appropriately and consistent with that hypothesis.

DR. GIANTURCO: Regarding the differences in B-48 and B-100, we've noticed that B-48 does not seem to be cleaved by thrombin. Has anyone else had any experience with that?

DR. BREWER: Yes. We find it to cleave.

DR. WEISGRABER: We've looked at it in βVLDL and it's not cleaved. Marcel and coworkers made B-48 βVLDL and we've tried to cleave it but we weren't able to.

DR. BREWER: Can you cleave this in the apoprotein?

DR. WEISGRABER: We haven't tried that.

DR. BREWER: That's what we've done, as an apoprotein, not in the lipoprotein particle.

DR. GIANTURCO: We find the contamination in the hypertriglyceridemic VLDL or in beta VLDL from cholesterol fed rabbits that it is not cleaved. It's a different conformation possibly.

DR. WITZTUM: Steve Young has done work in our laboratory which bears on this question. Because ApoB-37 is found in the HDL density range on a separate lipoprotein particle-- I guess Dr. Alaupovic would call it LPB-37--we've been able to isolate it and make an antisera. We took that approach so we would have multiple antibodies and this antisera reacts very well with the thrombin terminal fragment of the N-terminal region, but not the middle portion of B-100 and it doesn't react at all with the carboxy terminal fragment which would suggest that it is very comparable data to which that Dr. Havel has.

DR. BARTER: Dr. Krauss, about the poly-dispersity versus the pauci-dispersity concept, have you investigated these in vitro using the combination of the lipid transfer and the lipases to look at inter-conversions and see whether there is a sudden flip from one subpopulation to the next rather than a continuum and, it so, what is doing that?

DR. KRAUSS: We've done a partial degree of triglyceride enrichment of IDL-1. We have not fully substituted the triglyceride, but we've taken most of the cholesterol-rich IDL and converted it to triglyceride, but not all of it. We find that, when we incubate it with protein lipase, there are indeed two distinct products. We have presumably two subpopulations of precursor particles, one that is triglyceride enriched and one of which remains cholesterol enriched and we see two products in different proportions, but discrete products.

So, we feel that there is a quantum transition that appears to occur. We actually have an idea as to how that might occur since Tom Musliner in our laboratory has found that there are physical complexes that appear to occur between partially lipolized VLDL and LDL and other lipoproteins and it is possible, in our view, that a complex is formed between two individual particles that facilitates individual transfer which is kinetically a very rapid process and results in a subpopulation of particles being totally converted from cholesterol to triglyceride and another subpopulation that remains cholesterol enriched.

SPEAKER: Dr. Krauss I just wondered it part of the non-overlapping nature of your spectrum that you're seeing is due to the presence of only lipoprotein lipase and not hepatic lipase. In my hands very recently, looking with respect to E-containing lipoproteins, it is very clear that I get a different profile of E-containing lipoproteins when in vitro I expose them to partially purified LPL versus HTGL. The E-containing VLDL are very nice substrates for the lipoprotein lipase, but in effect build up the E-containing IDL, whereas the HTGL leaves the VLDL-containing E alone and will modify the content of the IDL.

DR. KRAUSS: We've certainly considered that. You're talking about purified human post-heparin plasma?

SPEAKER: Yes.

DR. KRAUSS: We found that when we used sufficiently high activities for purified bovine milk LPL that the properties are theres We simply overload the system with its hydrolytic capacity. We haven't specifically looked at all of the incubation conditions that you have described, but we feel that with bovine milk LPL you can override any distinctions between LPL and HTGL.

SPEAKER: With the E-containing lipoproteins anyway you can actually see--it is as if it is stopping there and building up.

DR. KRAUSS: That may be physiologically very important.

DR. DECKELBAUM: In vitro, when we manipulate different kinds of systems, certainly we see changes in the apoprotein content of the triglyceride-rich LDL-like particles we modify. For example, just incubating LDL with lipoprotein-deficient plasma, you'll find small amounts of ApoE, Al perhaps, on the LDL. If you enrich the LDL with triglyceride, with transfer protein, you again find ApoE. We have used overloaded systems with lipases, but now we are starting experiments with the two lipases and we find subtle differences. So, I'm wondering if in your two parallel

schemes, you might want to perhaps postulate the influence of apoproteins, different lipases, i.e. that they may overlap a little more than you think.

DR. KRAUSS: That's entirely possible physiologically, yes.

SESSION II. Heterogeneity of Triglyceride-Rich Lipoproteins Assessed *In Vitro*

Chair: Alan Fogelman
Cochair: Paul Nestel

VERY LOW DENSITY LIPOPROTEIN HETEROGENEITY IN NORMAL AND HYPERTRIGLYCERIDEMIC SUBJECTS

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Introduction

Very low density lipoproteins (VLDL) (d < 1.006) are heterogeneous in size (25-80 nm diameters), particle distribution, composition, density (S_f20-400), cellular receptor interactions, and metabolic fate. In terms of composition, VLDL are heterogeneous in lipid content (cholesteryl ester-rich versus triglyceride-rich) and in apoprotein content, conformation, and fragmentation. ApoB-containing lipoproteins range as a precursor-product continuum from the largest, lightest, triglyceride-rich VLDL to small, dense cholesteryl ester-rich low density lipoproteins (LDL).The largest VLDL contain ~170 moles of soluble apoC peptides and minimally one mole of apoE in addition to one mole of apoB whereas LDL contain apoB exclusively. VLDL from different subjects, particularly normal versus hypertriglyceridemic subjects, are also heterogeneous in terms of particle distribution, with normal subjects generally having mostly small, cholesteryl ester-rich VLDL and few large, triglyceride-rich VLDL. Triglyceride hydrolysis in VLDL by lipoprotein lipase is accompanied by transfer of water-soluble apoproteins and phospholipid to HDL; during this process most large VLDL are converted into small VLDL. Intermediate density lipoprotein subspecies (IDL) are derived primarily from small VLDL and LDL subspecies in turn are derived primarily from IDL. Metabolic studies indicate that not all large VLDL are converted into LDL, however. In hypertriglyceridemia, as much as two-thirds of total VLDL are not converted to LDL but are lost directly from the plasma compartment (4). Cell culture studies have provided potential cellular mechanisms to explain the direct loss of VLDL from plasma (5-10). Large VLDL (S_f 60-400) from hypertriglyceridemic (HTG) subjects, in sharp contrast to VLDL S_f60-400 from normal subjects, bind to and are degraded via at least two distinct cell surface receptors: the classic LDL receptor (5-7), and a distinct receptor present on macrophages (8,9). These receptor-mediated pathways, originally described by Goldstein and Brown and colleagues (11,12), afford at least two potential cellular routes for the direct catabolism of VLDL S_f60-400 in hypertriglyceridemic subjects; these receptor-mediated routes are not available for VLDL S_f 60-400 in normal subjects. Uptake by these receptor-mediated routes provides a cellular mechanism for the observed direct loss of VLDL apoB from plasma in hypertriglyceridemic subjects (4 and C. Packard, this volume).

Total VLDL Particle Heterogeneity.

Because of their extreme heterogeneity, metabolic and cell culture studies using unfractionated VLDL are often ambiguous and difficult to interpret. Traditionally, total VLDL have been designated as the d < 1.006 fraction of plasma. A great percentage of the protein in the "total VLDL" isolated in a single ultracentrifugal step in an angle head rotor is not VLDL protein at all, particularly in VLDL from normal subjects. In hypertriglyceridemic subjects an average of 57 ± 13% (n=6) of the protein in the d < 1.006 fraction is VLDL protein; the remainder of the protein (43 % ± 12%) consisted primarily of IDL, but other plasma proteins and LDL contaminate this fraction. (5,13,14)

The d < 1.006 fraction obtained by flotation and "total VLDL" isolated by gel filtration chromatography from normal plasma contain IDL, as shown by rate zonal ultracentrifugation (Figure 1) (5). The presence of IDL explains why "total VLDL" from normal subjects, in contrast to purified normal VLDL S_f 60-400, can cause receptor-mediated HMG-CoA reductase suppression due to uptake of the small VLDL by the LDL receptor pathway (5,7,15). "Total VLDL" (d < 1.006) from normal fasting subjects contain few large triglyceride-rich lipoproteins (S_f 60-400): only approximately one-third of the particles are S_f 60-400 and two-thirds are the cholesteryl ester-rich S_f 20-60 in normal subjects (13). The opposite is generally true of total HTG-VLDL: two-thirds are triglyceride-rich VLDL S_f 60-400 while one-third are cholesteryl ester-rich S_f 20-60 (13). Comparison of compositions of total HTG-VLDL vs. total normal VLDL, even after eliminating contaminating IDL, therefore gives the false impression that HTG-VLDL particles are enriched in triglyceride in comparison to normal VLDL. Comparison of the total compositions of more homogeneous subfractions of VLDL obtained by cumulative flotation (16) reveals that normal VLDL subclasses are qualitatively similar to HTG-VLDL subclasses in major components, but the distribution of particles is different. The composition of normal and hypertriglyceridemic VLDL subclasses of the same flotation rate are quite similar in terms of total lipid and protein content (14-16). VLDL subclasses from hypertriglyceridemic subjects are slightly enriched in cholesteryl ester relative to normal VLDL subclasses, as others have noted. This enrichment in cholesteryl ester is obscured in "total" HTG-VLDL. As mentioned above, HTG-VLDL was thought to be enriched in triglyceride compared to "total" normal VLDL. Likewise, the apoprotein content of normal VLDL and hypertriglyceridemic VLDL subclasses is qualitatively similar. Normal VLDL contain both apoB and apoE, yet $VLDL_1$ and $VLDL_2$ do not bind to the LDL receptor. When VLDL are subfractionated and contaminating lipoproteins removed by rate zonal ultracentrifugation(5) or by cumulative flotation (7,13,15), normal VLDL S_f 60-400 do not bind to the LDL receptor whereas HTG-VLDL S_f 60-400 do. This important functional distinction is due to subtle differences in both apoE content (as little as 2% of the protein mass) and in apoE conformation rather than qualitative differences in components.(2,13,14) These studies emphasize the importance in cell and metabolic studies to fractionate total VLDL (d < 1.006 fraction) into VLDL subclasses. As almost no one would now use total HDL, especially including HDL_1, the consequences of using total VLDL lead to false interpretation concerning metabolic fate, composition, and receptor ligand specificity.

In contrast to the functional distinctions between large VLDL S_f 60-400 from normal and hypertriglyceridemic subjects, small VLDL S_f 20-60 from both normal and hypertriglyceridemic subjects bind to the LDL receptor (7,15) This

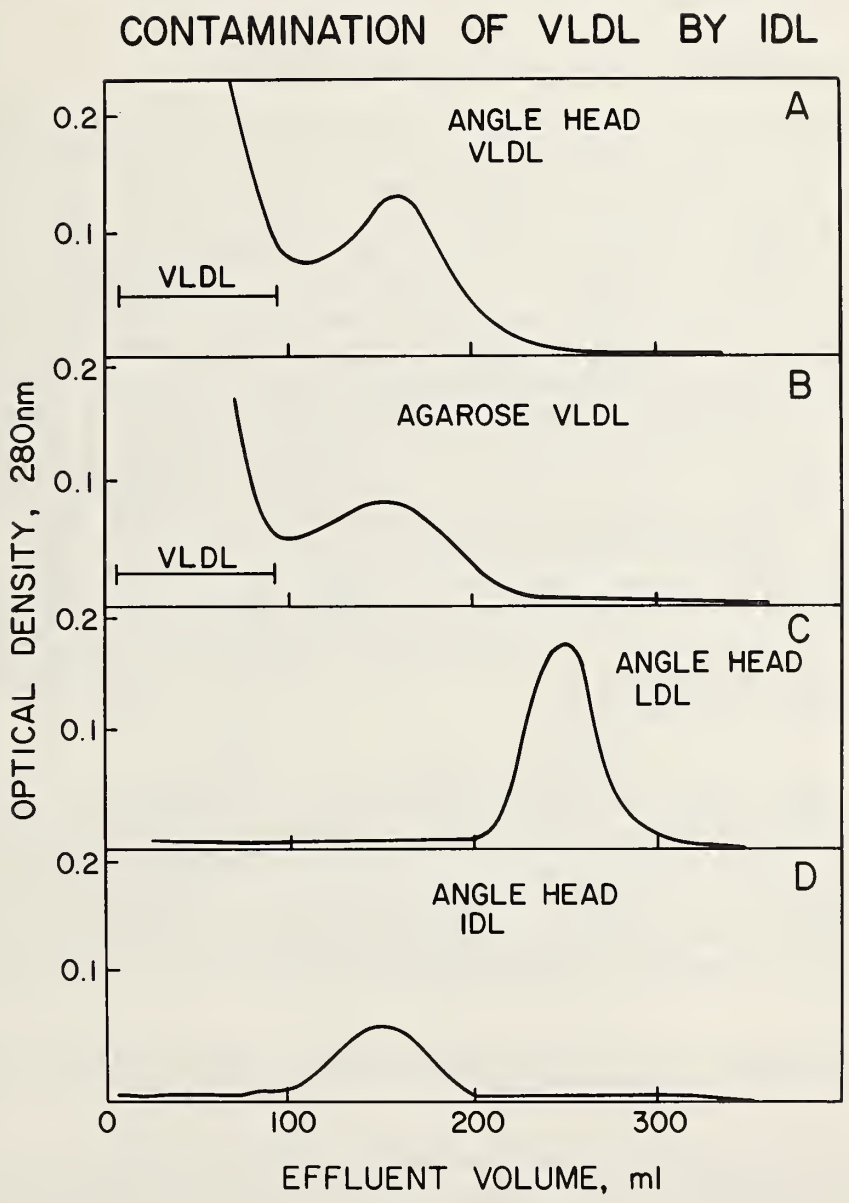

Figure 1. Contamination of d < 1.006 VLDL by IDL as determined by rate zonal ultracentrifugation. Normal VLDL isolated at d < 1.006 in an angle head rotor (A) and by gel filtration over BioGel A-5m (B) contain IDL. C shows the relative position of LDL (angle head rotor, d = 1.02-1.063), and D shows the relative position of IDL (angle head rotor d = 1.006-1.02) when these lipoproteins are recentrifuged in the zonal rotor. Fractions in the barred area of A and B are VLDL. Only the first 400 ml of the total rotor volume (665 ml) was collected (5).

provides a cellular catabolic route for direct loss of small VLDL from plasma in normal as well as hypertriglyceridemic subjects, which appears to occur in vivo (Packard, this volume).

Heterogeneity in binding of VLDL to the LDL receptor:

One criterion for specific binding of a lipoprotein to the LDL receptor is that binding curves demonstrate saturability, that is curvilinearity rather than linearity. As can be seen by the lower curve of Figure 2, normal $VLDL_1$ and $VLDL_2$ (S_f 60-400) bind in a linear rather than a curvilinear fashion to cultured normal human fibroblasts, indicating the binding is low affinity and nonspecific. By contrast, $VLDL_3$ (S_f 20-60) bind to normal human fibroblasts in a high affinity, curvilinear, saturable manner, as illustrated by the top two curves of Figure 2. Another criterion for specific binding to the LDL receptor of fibroblasts is uptake and accumulation within the cell at 37° versus 4°. As can be seen in the lowermost curve of Figure 2, the amount of $VLDL_1$ and $VLDL_2$ associated with the cells at 37° was equal to and not greater than that that bound to the cells at 4°, indicating that these lipoproteins were not being taken up by the cells. By contrast, the smallest VLDL subclass, $VLDL_3$, are taken up to a greater extent at 37° than at 4° (Figure 2). As a consequence of the inability of normal $VLDL_1$ and $VLDL_2$ to bind to and be internalized via the LDL receptor, these VLDL do not suppress HMG-CoA reductase activity (Figure 3). By contrast, the smallest $VLDL_3$ subclass from normal subjects does have the ability to cause receptor-mediated HMG-CoA reductase suppression (Figure 3).

In contrast to the VLDL subclasses from normal subjects, all hypertriglyceridemic VLDL subclasses bind to the LDL receptor, as can be seen in Figure 4. Direct binding studies at 4° show that HTG-$VLDL_1$, HTG-$VLDL_2$, and HTG-$VLDL_3$ have similar affinities for the LDL receptor of cultured human fibroblasts (Figure 4). Again, as a consequence of the ability of these three VLDL subclasses to bind to the LDL receptor, each of these VLDL subclasses has the ability to cause receptor-mediated HMG-CoA reductase suppression, as shown in Figure 5.

Heterogeneity of apoE in VLDL from normal versus hypertriglyceridemic subjects.

Although the LDL receptor binds either apoE or apoB in small particles, apoB of large VLDL S_f 60-400 is not recognized by the LDL receptor (2,7) or by certain antisera against apoB (17), suggesting different conformational microdomains in VLDL relative to LDL. A particular conformation of apoE is absolutely required for the binding to and uptake of large VLDL S_f 100-400 by the LDL receptor (2,7). This conformation of apoE is generally present in HTG-$VLDL_1$ but not in normal $VLDL_1$ (3,13). In contrast to normal subjects, the distribution of the apoE in plasma in hypertriglyceridemic subjects is shifted to VLDL rather than HDL (18). Analogous to this transfer in vivo, exogenous apoE can be incorporated into normal S_f 60-400 VLDL in vitro, creating the LDL receptor recognition domain (7). We hypothesized that the apoE in HTG-VLDL S_f 100-400 required for binding to the LDL receptor transfers into the VLDL during its longer residence time in hypertriglyceridemic subjects (2). This apoE is accessible not only as a ligand for the LDL receptor but as a substrate for peptide bond cleavage by thrombin as well: thrombin cleaves specifically the apoE in HTG-VLDL which binds to the receptor, thus abolishing

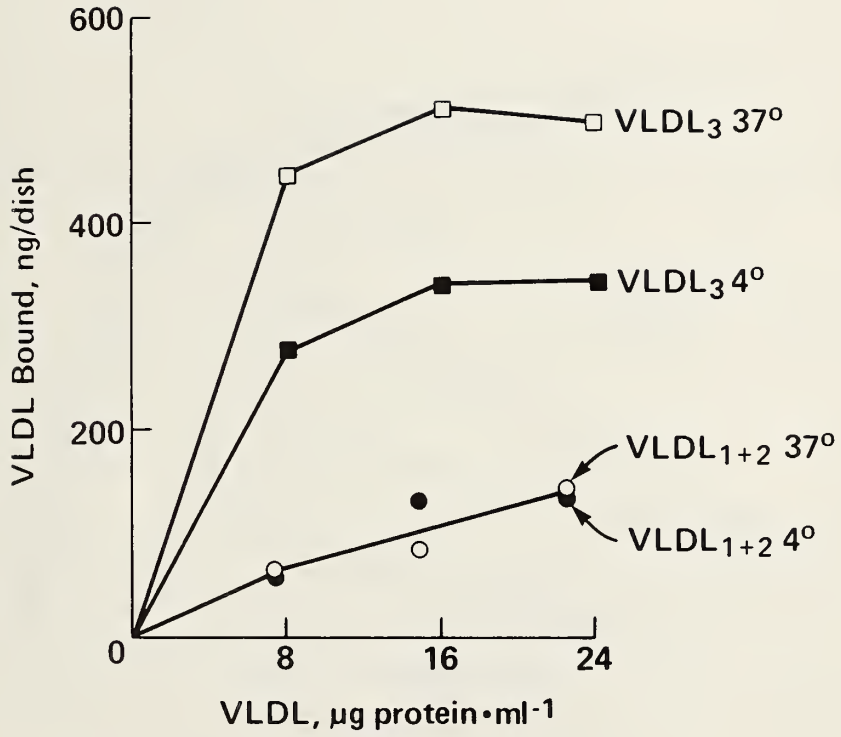

Figure 2. Uptake of normal VLDL subclasses by normal human fibroblasts. Cells were grown in 60-mm dishes in complete medium for 4 days, preincubated for 48 hr in medium containing 5% lipoprotein-deficient serum, and incubated for 2 hr with the indicated quantities of [^{3}H]triolein-labeled S_f 60-400 VLDL (○ ●) or S_f 20-60 VLDL (□ ■) at 37°C (open symbols) or 4°C (closed symbols). The cells to be incubated at 4°C were precooled for 30 min prior to addition of the lipoproteins. The cells were washed extensively with albumin-containing buffer, dissolved in 1 ml of 1% sodium dodecyl sulfate for liquid scintillation counting. Each point represents the average of values of duplicate dishes (variation ≤ 10%). The average protein content per dish was 424 µg.

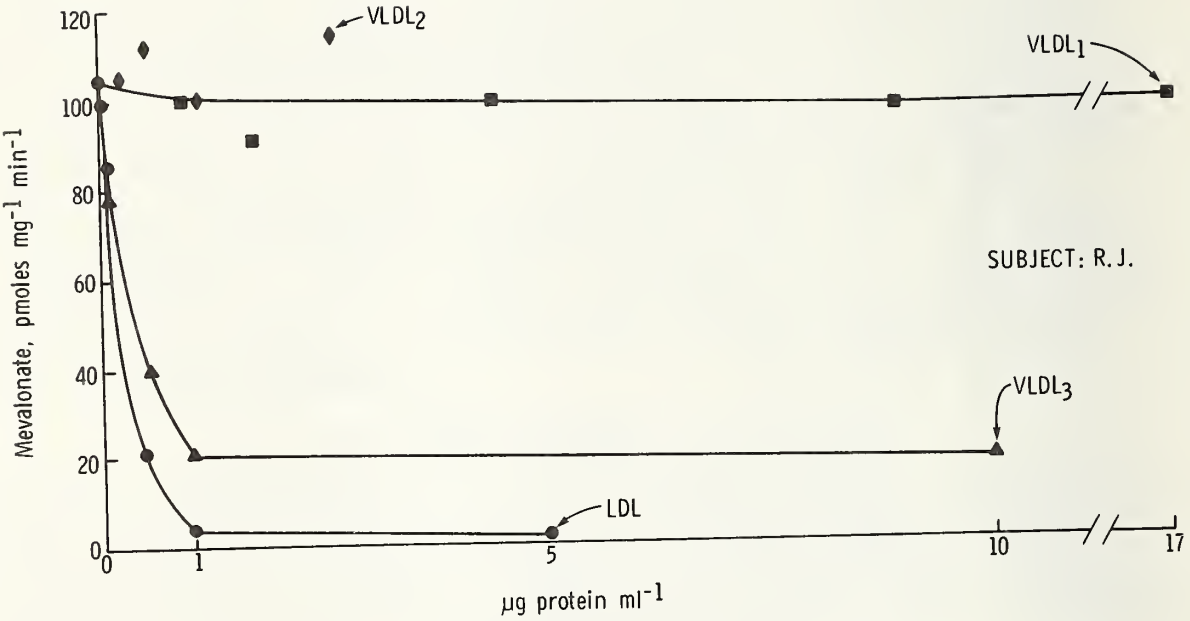

Figure 3. Effects of normal VLDL subclasses and LDL on HMG-CoA reductase activity in normal human fibroblasts. Cells were grown to approximately 75% confluency in complete medium containing 10% fetal calf serum, washed and placed on 2 ml of medium containing 5% LPDS for 24 hr. Indicated amounts of lipoproteins, in 0.2 ml, were added to duplicate dishes for 16 hr before the cells were washed and harvested. Each data point is an average of duplicate determinations of HMG-CoA reductase activity in cells from duplicate dishes. Normal LDL (●); normal fasting $VLDL_1$ (■); $VLDL_2$ (◆); and $VLDL_3$ (▲), purified by flotation through a density gradient d 1.006-1.05 (15,16).

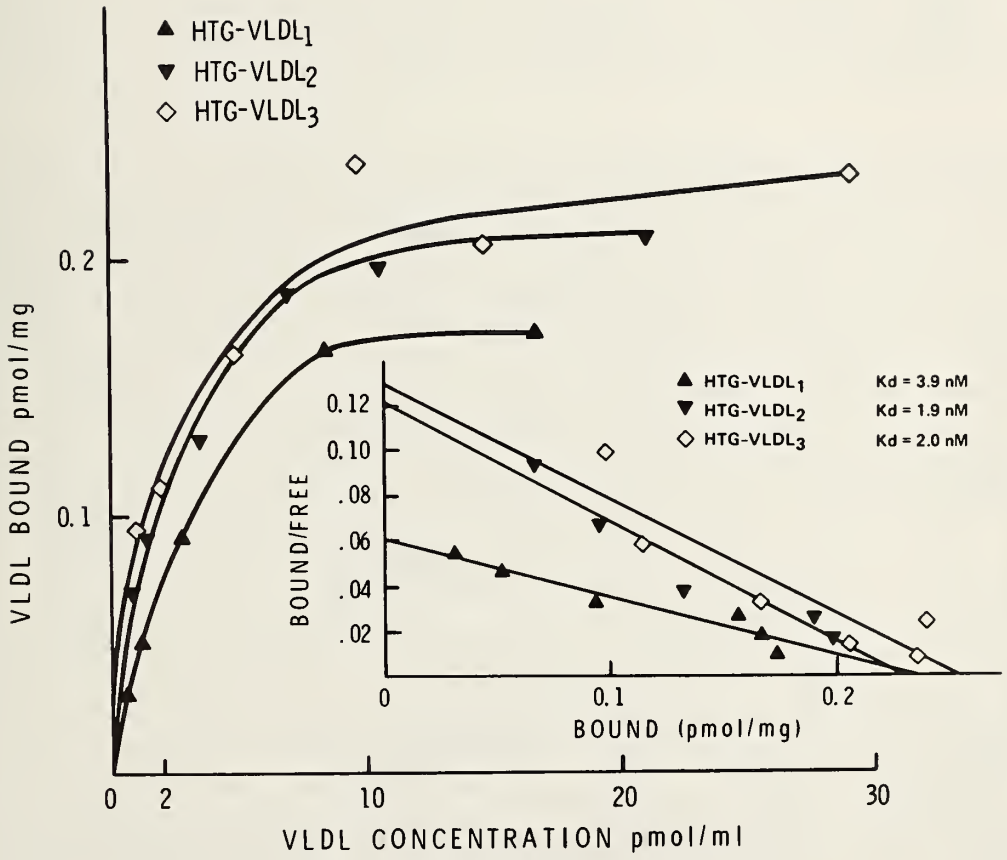

Figure 4. Specific binding curves of HTG-VLDL$_1$, HTG-VLDL$_2$, and HTG-VLDL$_3$ indicate similar binding affinities. Indicated quantities of ^{125}I-HTG-VLDL subclasses alone and with excess unlabeled homologous VLDL were incubated for 3 hr at 4°C with normal fibroblasts which had been preincubated in lipoprotein-deficient medium. For calculation of particle masses from determined protein concentrations, the following values were used: 30 X 10^6 daltons/HTG-VLDL$_1$ (6% protein); 17 X 10^6 daltons/HTG-VLDL$_2$ (8.4% protein); 10 X 10^6 daltons/HTG-VLDL$_3$ (10.3% protein). Each point represents specific binding values in terms of picomoles of lipoprotein/mg of cell protein plotted versus lipoprotein concentration (picomoles/ml). The data are linearized by Scatchard's method to obtain apparent K_d values in terms of picomoles of lipoprotein/ml (inset).

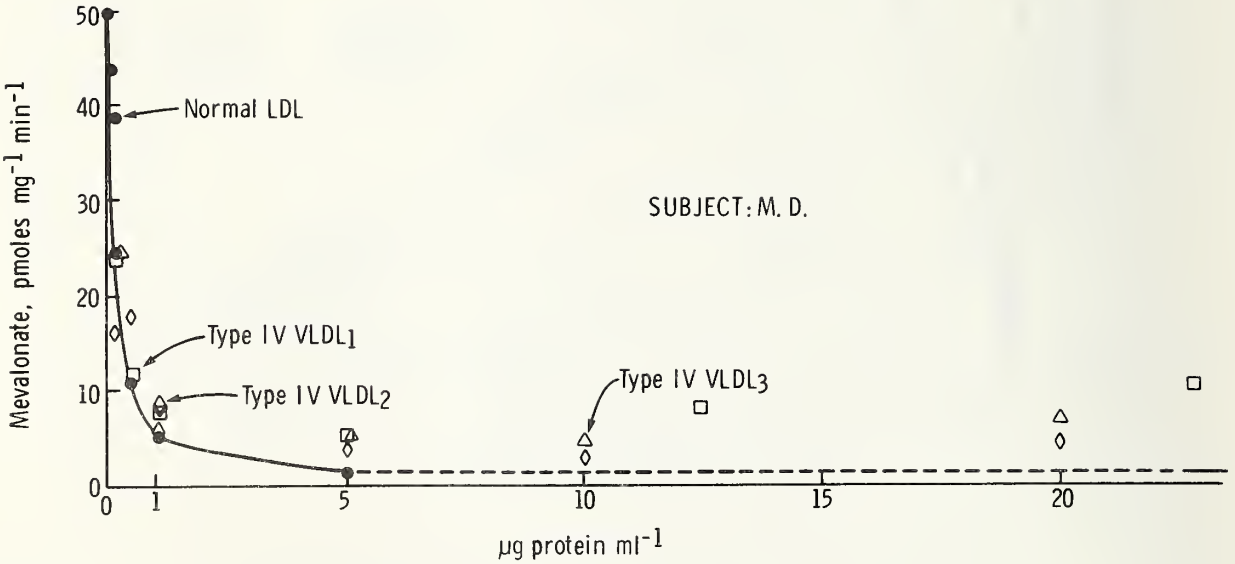

Figure 5. Effects of Type IV hypertriglyceridemic VLDL subclasses on HMG-CoA reductase activity in normal fibroblasts. Growth of the cells and the experimental design were the same as described in the legend to Figure 3. Each data point is the average of duplicate determinations of HMG-CoA reductase activity in cells from duplicate dishes. The VLDL were isolated from the plasma of a subject with primary Type IV hyperlipoproteinemia. Normal LDL (●); $VLDL_1$ (□); $VLDL_2$ (◇); and $VLDL_3$ (△) were isolated by cumulative flotation (15,16).

its binding; other apoE molecules on the same HTG-VLDL remain intact, indicating that the thrombin-resistant apoE molecules are conformationally inaccessible both to thrombin and to the LDL receptor (2). Therefore, another heterogeneity in VLDL exists in terms of apoE conformation. Unlike HTG-VLDL, generally all of the apoE of $VLDL_1$ from normal subjects is inaccessible both to the LDL receptor (7) and to thrombin (3). This inaccessible conformation of apoE is present in both normal and HTG-VLDL (1 to 2 moles/particle). We suggested that the inaccessible apoE conformation is in nascent, hepatic VLDL ("endogenous" apoE), and that the accessible apoE conformation present in HTG-VLDL may transfer into the VLDL after it reaches the plasma compartment (1 to 4 moles of "exogenous" apoE) (2).

Thrombin hydrolyzes isolated apoE or the accessible apoE in HTG-VLDL primarily at one out of eight potential cleavage sites (the Arg-Ala bond at residues 191-192), producing two major fragments termed E-12 and E-22 (1,2). E-22 is the N-terminal two-thirds and contains a domain which binds to the LDL receptor (19). E-12 is the C-terminal one-third and contains a primary lipid binding region of the molecule. Even without removal of the E-22 fragment, thrombin cleavage abolishes or significantly reduces the binding of HTG-$VLDL_1$ to the LDL receptor and, consequently, its suppression of HMG-CoA reductase activity (2). Reincorporation studies show that this loss of biological activity was due to the cleavage of apoE: reincorporation of intact apoE into thrombin-inactivated HTG-VLDL S_f 100-400 (~1 mol/mol particle) restores efficient receptor-mediated reductase suppression. Addition of the E-12 fragment failed and the E-22 fragment was on a molar basis only 50% as effective as intact apoE in restoring the ability of thrombin-inactivated HTG-$VLDL_1$ to suppress reductase activity. The E-12 domain appears involved in maintaining apoE in the proper conformation for optimal receptor recognition for binding of HTG-$VLDL_1$. At least one mol/mol VLDL of intact apoE of the thrombin-accessible conformation is required for binding of HTG-$VLDL_1$ to the LDL receptor. Although thrombin hydrolyzed apoB, thrombin produced no change in the immunoreactivity of apoB of HTG-$VLDL_1$ nor affected the ability of LDL (as control) to bind to the LDL receptor or suppress HMG-CoA reductase (2). Retention of biological activity of LDL after proteolysis is well documented (20). ApoB-mediated binding ($VLDL_3$, IDL,LDL), in contrast to apoE-mediated binding, is not affected by thrombolysis and the inactivation of HTG-$VLDL_1$ by thrombin was due to its effect on apoE and not due to an effect on apoB.

VLDL Receptor Binding Determinant Heterogeneity.

The binding determinants for large lipoproteins are clearly different from those of small lipoproteins: apoE of the thrombin-accessible conformation is necessary and apoB insufficient for uptake of $VLDL_1$ whereas apoB alone is sufficient for receptor binding of LDL. Heterogeneity in binding determinants for the LDL receptor represents another form of heterogeneity in particles in the VLDL spectrum.

In the metabolic continuum from HTG-$VLDL_1$ to LDL, binding determinants for the LDL receptor switch from apoE to apoB. Studies with thrombin and trypsin to inactivate apoE but not apoB as a ligand for the LDL receptor (2,13,14) demonstrate that apoE of the thrombin-accessible conformation is required for the receptor binding of HTG-$VLDL_1$, HTG-$VLDL_2$, and a small population of HTG-$VLDL_3$, but not for the receptor binding of most of HTG-$VLDL_3$, IDL or LDL (13). The shift from apoE to apoB as the primary LDL receptor binding

determinant in the hypertriglyceridemic $VLDL_1$ to LDL series therefore occurs within the same region in the metabolic cascade where one first observes binding, uptake and degradation of lipoproteins from subjects with normal plasma triglycerides: within the smallest VLDL subclass of S_f 20-60 (7,13,15). These conclusions have been confirmed using monoclonal antibodies against apoE and apoB (10, Krul, this volume).

The apoB in the larger VLDL (S_f 60-400) appears to be in an inappropriate conformation for receptor binding. With the decrease in surface area that occurs upon lipolysis, the conformation of apoB changes and such a change appears to be required before apoB can interact with the LDL receptor (10,17,20,21). The inability of apoB to mediate uptake of large VLDL (and chylomicrons, chylomicron remnants) by the LDL receptor pathway could serve to protect cells from the potentially toxic effects of uptake of triglyceride-rich particles by extrahepatic cells. The production of intracellular fatty acids upon lysosomal hydrolysis of the LDL receptor HTG-VLDL may be responsible for the observed toxicity of HTG-$VLDL_1$ that is not observed with normal $VLDL_1$ in endothelial cells and may cause endothelial injury if it occurs in vivo (22).

These studies indicate apoB normally is the physiologically important ligand for the LDL receptor (14,19). Even though native IDL and $VLDL_3$ contain apoE, and even LDL contain traces of apoE, these studies indicate that apoB normally mediates binding of most of the $VLDL_3$, IDL and LDL to the LDL receptor, as originally proposed by Brown and Goldstein (23).

We suggest that apoE only plays a role in the binding of lipoproteins to the LDL receptor in abnormal, pathologic situations such as hypertriglyceridemia, abetalipoproteinemia, or hypobetalipoproteinemia. The redundancy in receptor recognition could be beneficial in hypo- or abetalipoproteinemia, in which HDL containing apoE rather than LDL could deliver cholesterol to cells in vivo. The ability of apoE, in contrast to apoB, to bind to the receptor in large as well as small particles could be beneficial in hypertriglyceridemia, in which the abnormal LDL-receptor-mediated uptake of large HTG-VLDL may be an alternate route for degrading the excess VLDL that are not lipolized efficiently.

Conclusions

VLDL are extremely heterogeneous both functionally and structurally, but more homogeneous subclasses can be isolated by cumulative flotation. Studies comparing "total" VLDL with VLDL subclasses so isolated (this paper and Packard, this volume) strongly suggest that use of total VLDL in cell culture and in vivo metabolic studies can lead to critical misconceptions. Both metabolic and cell studies indicate that large triglyceride-rich VLDL (S_f 60-400) are functionally (metabolically) as well as compositionally distinct from small cholesteryl ester-rich VLDL (S_f 20-60). Since "total" normal VLDL ($d < 1.006$) are predominantly the small VLDL S_f 20-60 and "total" hypertriglyceridemic VLDL are predominantly the large, triglyceride-rich VLDL, direct comparisons of total normal VLDL to total HTG-VLDL can be as ambiguous as comparing apples and oranges. At present, subfractionation of VLDL by cumulative flotation (16) or rate zonal ultracentrifugation (24) appear to be the methods of choice, since these are at present the most practical and certainly the best characterized methods of obtaining more homogeneous VLDL

subclasses. As presented at this conference, however, methods based on apoprotein content and conformation (Alauopovic, Krul, this volume), may also provide additional useful techniques for the subfractionation of VLDL based on functionally significant properties.

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RELATION OF ApoB/ApoE EPITOPE EXPRESSION TO LDL RECEPTOR BINDING

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Very low density lipoproteins (VLDL) contain both apolipoproteins B and E on their surfaces. ApoB and apoE both interact with the cellular low density lipoprotein (LDL) (or apoB,E) receptor. But the mere presence of apoB and apoE on the lipoprotein particles appears not to be sufficient for receptor recognition. The exposure of apoB and/or apoE molecules on the surfaces of VLDL particles (an example of VLDL structural heterogeneity) appears to modulate the rates of clearance of triglyceride-rich lipoproteins from plasma in vivo and by cultured cells in vitro (1-5). Thus the conformations of apoproteins on lipoproteins could be important in regulating the concentrations of atherogenic lipoproteins in plasma and interstitial fluids (3,6).

A simplified model of VLDL structural heterogeneity based on apoB/apoE epitope expression is shown in Fig. 1. This model arose from studies by our group and by others employing immunochemical (7-9) and enzymic reagents (10,11) as probes of the conformations of apoB and apoE on the surfaces of lipoprotein particles.

Studies conducted several years ago in our laboratory demonstrated that VLDL lipolyzed in vitro or smaller $VLDL_3$ were better competitors of ^{125}I-LDL binding to fibroblast LDL receptors than the starting VLDL preparation or the large $VLDL_1$ subfractions isolated from plasma (7). Parallel changes were noted in the immunoreactivity of apoB as determined with polyclonal antisera, i.e. lipolyzed VLDL and $VLDL_3$ were more immunoreactive than unlipolyzed VLDL and $VLDL_1$. It appeared that apoB became more "exposed" to both the antisera and the LDL receptor during lipolysis of VLDL.

The importance of apoE in mediating the receptor recognition of VLDL particles was demonstrated by Gianturco and co-workers (10). They reported that enzymatic cleavage of apoE on large $VLDL_1$ particles isolated from hypertriglyceridemic (HTG) individuals significantly reduced the uptake of those particles by fibroblasts. The same group also showed that apoE on the surface of large HTG-$VLDL_1$ was accessible to enzymatic cleavage by thrombin or trypsin whereas apoE associated with smaller HTG-$VLDL_3$ or normolipidemic VLDL particles was not accessible to these enzymes (11). These studies indicated that some apoE molecules exist in an "exposed" conformation on larger VLDL particles and thus could mediate receptor recognition of these particles.

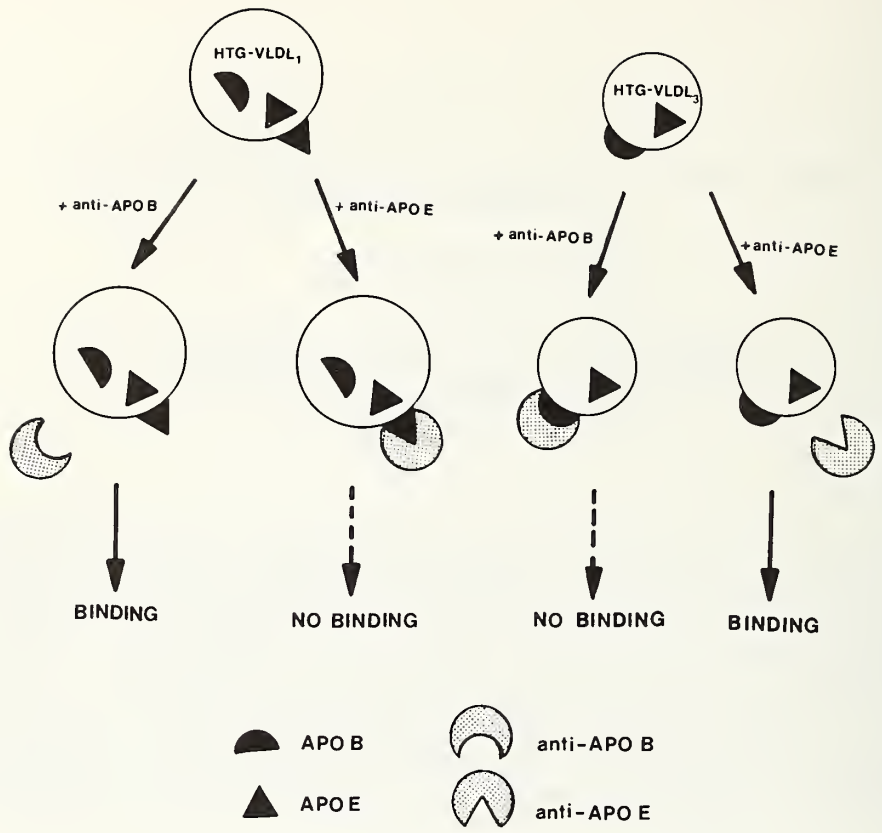

Figure 1. Model of VLDL structure based on ApoB and ApoE epitope expression. VLDL subfractions shown are $VLDL_1$ and $VLDL_3$ from hypertriglyceridemic individuals. The large $VLDL_1$ and small $VLDL_3$ can be prepared by density gradient or zonal ultracentrifugation. The presence of **accessible forms of apoB or apoE** on the VLDL particles is necessary and sufficient for mediating cellular LDL receptor binding. ApoB associated with large HTG-$VLDL_1$ is for the most part inaccessible (drawn inside the VLDL particle) to the LDL receptor whereas on smaller $VLDL_3$ apoB is mostly in an accessible conformation (shown on the outside of the particle). The converse is true for apoE. Most accessible forms of apoE exist on the surfaces of large HTG-$VLDL_1$. The apoE on small VLDL is inaccessible. See text for details.

Experiments conducted in our laboratory using monoclonal anti-apoB and anti-apoE antibodies to inhibit VLDL binding and intracellular processing by cultured fibroblasts confirmed our initial experiments (7) and provided new insights about the dispositions of apoB and apoE on VLDL particles (Fig.1) (8). Thus a MAb directed against the receptor recognition region of apoB was inefficient at inhibiting the cellular processing of large HTG-$VLDL_1$ (Fig. 2), while an anti-apoE MAb did inhibit the cellular processing of $VLDL_1$. Conversely, the uptake of smaller $VLDL_3$ was not inhibited by the anti-apoE antibody, but the anti-apoB antibody was an increasingly effective inhibitor of binding of VLDL to the apoB,E-receptor as the VLDL particles became denser and smaller in size.

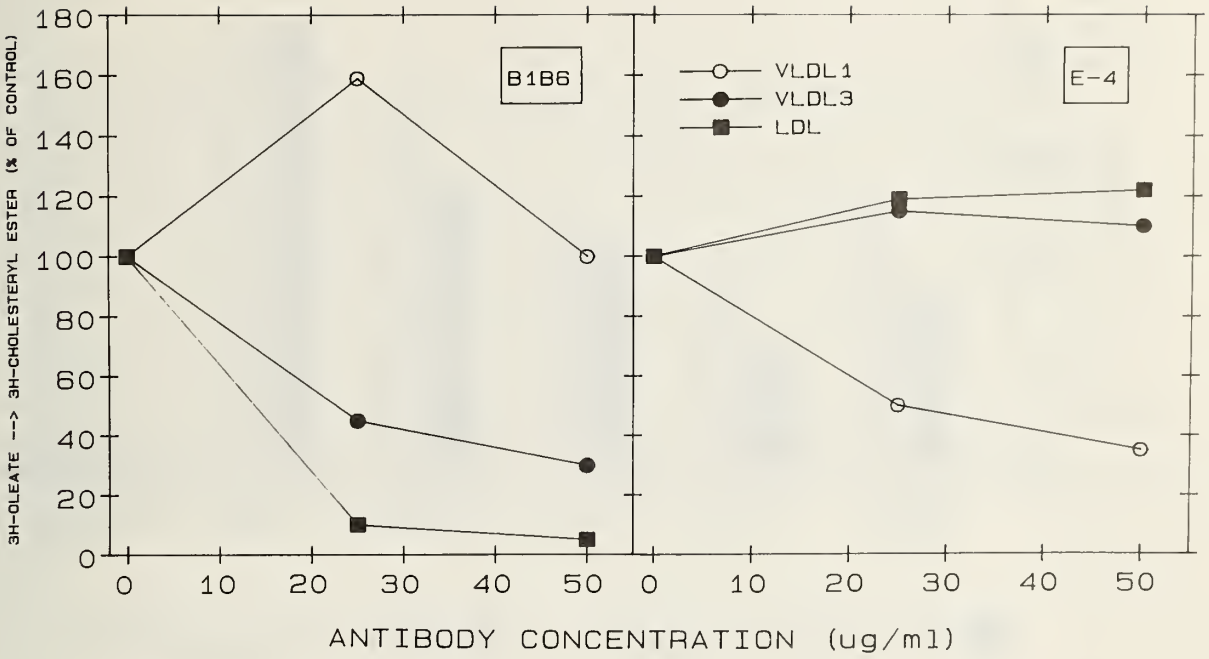

Figure 2. Monoclonal antibodies inhibit lipoprotein uptake-dependent esterification of cholesterol by fibroblasts. Experiments showing inhibition of VLDL-dependent esterification by the anti-apoB MAb (B1B6) are shown in the left panel and by anti-apoE MAb (E-4), in the right panel. Fibroblasts were cultured in a medium containing lipoprotein-deficient serum for 48 hr prior to the addition of lipoproteins either alone or in the presence of the indicated amounts of purified MAb. Lipoproteins were isolated from plasma by sequential density ultracentrifugation (12) and the VLDL subfractions were separated by zonal ultracentrifugation (13). The amount of ^{3}H-oleate incorporated into cellular cholestryl esters was determined as previously described (9). Results are expressed as a percent of ^{3}H-oleate incorporation into esters in the absence of MAb.

The concept that the receptor recognition site of apoB is for the most part "masked" in the larger VLDL particles is also supported by affinity chromatography data from our laboratory (Fig.3). The immunoaffinity column was constructed using the MAb directed against the apoB,E receptor recognition regions of apoB-100. For all VLDL fractions the percent of total counts bound by the column was less than the percent of total counts precipitable by TMU. The proportion of total counts bound by the column increased as VLDL decreased in size. Virtually all LDL counts were bound. These results indicate that this apoB epitope is not accessible to antibody (or apoB,E receptor) in the large VLDL and that proportionately more VLDL particles express the epitope on their surfaces as the particle diminishes in size.

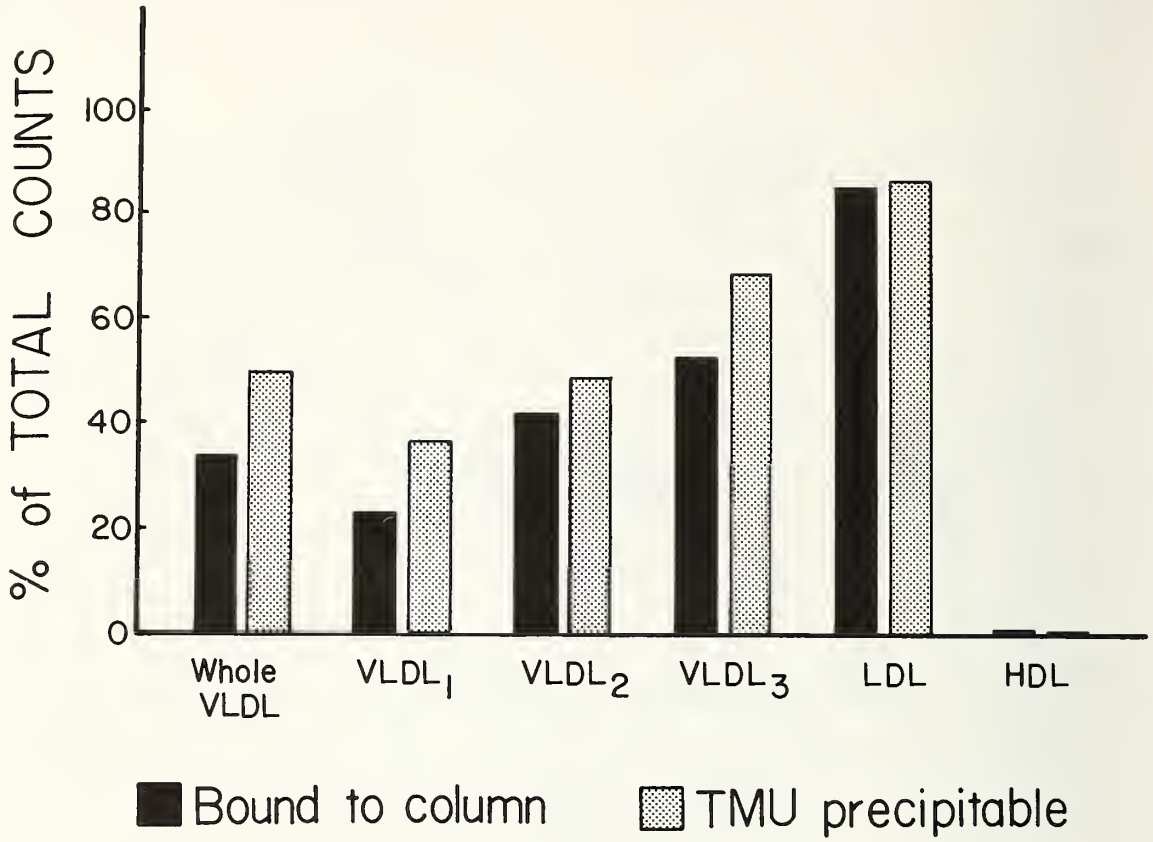

Figure 3. Immunoaffinity chromatography of lipoproteins on an anti-apoB column. Radiolabelled VLDL density subfractions, LDL and HDL were applied onto a Sepharose 4B CnBr column to which the monoclonal anti-apoB antibody B1B6 had been coupled. This antibody inhibits VLDL- and LDL-apoB binding to the cellular LDL receptor. The solid bars represent the percent of loaded counts bound by the column and the speckled bars represent the percent of total counts precipitable by tetramethylurea (TMU) (or the percent of counts in apoB). The VLDL fractions were isolated by zonal ultracentrifugation.

Despite our current knowledge of apoB/apoE epitope expression on VLDL particles, many questions still remain unanswered. Do all large HTG-VLDL$_1$ particles express apoE on their surfaces equally well or is this density subclass merely enriched in a population of particles whose apoE is particularly accessible while other particles contain either no apoE at all or only apoE inaccessible to antibodies? What are the factors responsible in determining the conformation of apoE or apoB on the surface of a lipoprotein particle?

We have begun to address these questions in our laboratory. Affinity chromatography of VLDL particles on anti-apoE MAb columns has been performed in order to determine what proportion of particles express specific apoE epitopes in accessible form. We then determine whether the availability of the epitopes to the antibodies correlates with the recognition of the VLDL particles by apoB,E-receptors. The unretained and retained particle compositions are then analyzed in order to determine whether compositional factors may be related to the binding of VLDL to the antibodies.

Immunoaffinity columns were prepared using anti-apoE MAbs, and VLDL (d<1.006) or VLDL subfractions were chromatographed. Lipid and apoprotein compositions of chromatographic fractions were analyzed. Both the retained and unretained fractions contained apoE, but apoE/apoB ratios of retained VLDL particles were higher. Next the interactions of the chromatographed VLDL fractions with cultured human fibroblasts were assessed in assays where VLDL competed with ^{125}I-LDL for binding to the LDL receptor. Retained fractions were several-fold more effective competitors than non-retained fractions. The data suggest a) that the availability of an epitope of apoE to antibody is closely related to its availability to the cellular LDL receptor, b) only certain subpopulations of VLDL contain apoE with available conformations, c) immunoaffinity chromatography may be an effective way of fractionating VLDL into functional subclasses, and d) immunologic means may be useful for quantifying the proportions of lipoprotein subpopulations with available epitopes present among apoE-containing lipoproteins.

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CATABOLISM OF TRIGLYCERIDE RICH LIPOPROTEINS IN TYPE 5 HYPERLIPOPROTEINEMIA: DIMINISHED *IN VIVO* CLEARANCE AND ENHANCED *IN VITRO* UPTAKE BY MACROPHAGES

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Abstract

The catabolism of triglyceride-rich lipoproteins (TRL) is highly heterogeneous. Particles of intestinal origin are normally removed from the plasma, as chylomicron remnants, at a much faster rate than the remnants of very low density lipoproteins (VLDL) which transport endogenously produced triglyceride. Recent studies have identified specific receptors and specific ligand-receptor interactions which differentiate between the two species of remnants. These findings have clarified understanding of the metabolic channelling of TRL.

We have examined the possibility that the marked hyperlipemia of Type 5 hyperlipoproteinemia reflects diminished lipolysis of TRL, leading to impaired ligand-receptor interaction. We have studied the *in vivo* clearance of TRL containing either apo B48 (chylomicron remnants) or apo B100 (VLDL remnants). The infusion of heparin accelerated the catabolism only of apo B100 TRL, demonstrating failure of chylomicrons to become degraded to the point where the particles can interact optimally with the hepatic chylomicron remnant receptor.

Since cells other than hepatocytes can metabolize TRL, we studied the *in vitro* catabolism of TRL from Type 5 hyperlipoproteinemic subjects. Incubations with J774 macrophages showed that in contrast to the *in vivo* findings, apo B48-rich TRL were taken up and degraded at a greater rate than apo B100-rich TRL or VLDL from hypertriglyceridemic subjects. Uptake occurred at sites that were indistinguishable from β-VLDL binding sites. The B/E or LDL receptor was not involved since monoclonal antibodies raised against apo B48, which prevented LDL uptake, did not inhibit TRL or β-VLDL catabolism. Moderate cholesterol enrichment of the macrophages was similar with TRL containing a 6-fold range of apo E, although TRL from subjects homozygous for E_2 were ineffective.

The *in vivo* and *in vitro* data suggest that the binding site for chylomicron remnants on macrophages is not the receptor present in liver.

The catabolism of triglyceride-rich lipoproteins (TRL) is highly heterogeneous. Particles produced in the intestine and transporting predominantly triglyceride of exogenous or dietary origin, undergo rapid intravascular catabolism under normal circumstances. As first shown by Nestel in 1964,[1] lymph chylomicrons reinjected into human subjects are removed at several times the rate of very low density lipoproteins (VLDL) produced in the liver. However, even among the latter, there is substantial variability in removal rates, reflecting differences in metabolic pathways. Recent studies have identified specific receptors and specific ligand-receptor interactions which have greatly improved our understanding of the metabolic channelling of TRL.

Chylomicrons and VLDL differ in size, in their lipid content and composition and in their constituent apoproteins.[2] Furthermore, the removal of their respective remnants is mediated by specific receptors which have now been isolated. Secreted chylomicrons rapidly acquire additional apoproteins from other circulating lipoproteins. Briefly, apo C_2 transfers from HDL to chylomicrons. Through a process which includes binding to the enzyme lipoprotein lipase, situated near the endothelial surface of capillaries, apo C_2 facilitates the lipolysis of chylomicron triglyceride. Other apoproteins, C_3 and E, which are also transferred from HDL, regulate other aspects of chylomicron catabolism. Apo C_3 probably prevents the premature removal of the catabolized chylomicron by hepatic receptors. Eventually, a remnant of the chylomicron is formed, through the removal of a critical fraction of triglyceride and a remodelling of the smaller residual particle. The remodelling includes the shedding of surplus surface material (apo A_1, A_2 and A_4 plus free cholesterol and phospholipid) and acquisition of some cholesteryl ester from HDL in exchange for chylomicron triglyceride. Apo E then mediates the recognition of the remnant by a hepatic receptor which is distinct from the B/E (LDL) receptor.

The chylomicron receptor is distinct from a receptor which recognises and binds LDL, although it interacts also with other apo E containing lipoproteins such as HDLc, e.g. from cholesterol-fed dogs. This receptor has been recently isolated from hepatocyte membranes: its molecular weight (56,000), primary structure and immunoreactivity differentiate it from the B/E (LDL) receptor in the liver.[3] Apo B48 which is the unique intestinal apo B does not appear to be involved in the ligand-receptor interaction. The observations made *in vitro* have been supported by *in vivo* studies in patients with homozygous familial hypercholesterolemia and in the similarly afflicted Watanabe hereditary hyperlipidemic rabbit. Despite the complete absence of LDL receptors, chylomicron clearance appears normal both in the FH patients and in the WHHL rabbit.[4] However, additional mechanisms for the removal of chylomicron remnants, including non-receptor mediated uptake and the involvement of other apoproteins and indeed of lipids have not been excluded.

The rapid removal of chylomicron remnants in man has been demonstrated by the very fast clearance of apo B48, the unique apoprotein of these particles,[5] as well as from the clearance of the lipid constituents. Radiolabelled fatty acids, incorporated mainly into the triglyceride within the core of the lipoprotein and radiolabelled retinyl palmitate which is also packaged into the core have shown similarly rapid removal rates for chylomicron remnants, measured in minutes in healthy normolipidemic subjects.[6] Chylomicron remnant clearance is inversely correlated with the fasting triglyceride concentration.[1]

The removal of VLDL remnants is largely a function of the LDL or B/E receptor, occurring mainly in the liver and involving both the B and E apoproteins. The removal of larger VLDL is mediated through apo E, whereas apo B becomes the ligand with increasing delipidation of the particle.[7] Ligand receptor binding therefore requires a critical degree of delipidation which allows the necessary degree of exposure or conformational change of the ligand. This is also evident in chylomicron catabolism which is hindered by surplus apo C3,[8] and possibly by apo A4, both of which are shed during lipolysis permitting apo E to interact with the receptor.

We have therefore examined the possibility that the hyperlipemia of Type 5 hyperlipoproteinemia reflects diminished lipolysis of the accumulated TRL, leading to impaired ligand-receptor interaction. The hyperlipoproteinemia in this disorder is characterized by the retention in the plasma of both intestinally derived and hepatogenous TRL. We had previously observed in these subjects a slower removal of those large particles characterized by

the intestinal apoprotein, apo B48, than of particles that contained apo B100,[9] considered a marker of VLDL. This suggested a slower removal of chylomicron remnants than of VLDL remnants which was surprising in view of the more rapid disappearance of apo B48 rich lipoproteins in normal subjects.[5]

We have therefore carried out studies in 4 subjects with primary Type 5 hyperlipoproteinemia to measure the comparative removal rates of chylomicron remnants and of large VLDL when lipolysis was stimulated by infusing heparin. The studies were performed over 7-8 hours. ^{125}I-labelled TRL (Sf>60) were reinjected and the decline in the radioactivities of apo B48 and of apo B100 determined over the first 3 hours. Sodium heparin was then infused at a constant rate of 2000 IU per hour (after a priming infusion) and maintained for the next 3 hours (90 min in subject 4). Changes in the rates at which apo B48 and apo B100 radioactivities disappeared from the Sf>60 fraction and appeared in the Sf12-60 lipoproteins were monitored during the infusions and after the heparin was stopped.

Apo B48 and apo B100 were separated by electrophoresis on 3.5% polyacrylamide gels. Total lipase and heparin triglyceride lipase activities were assayed in plasmas taken before and 30 min after beginning the heparin infusion.

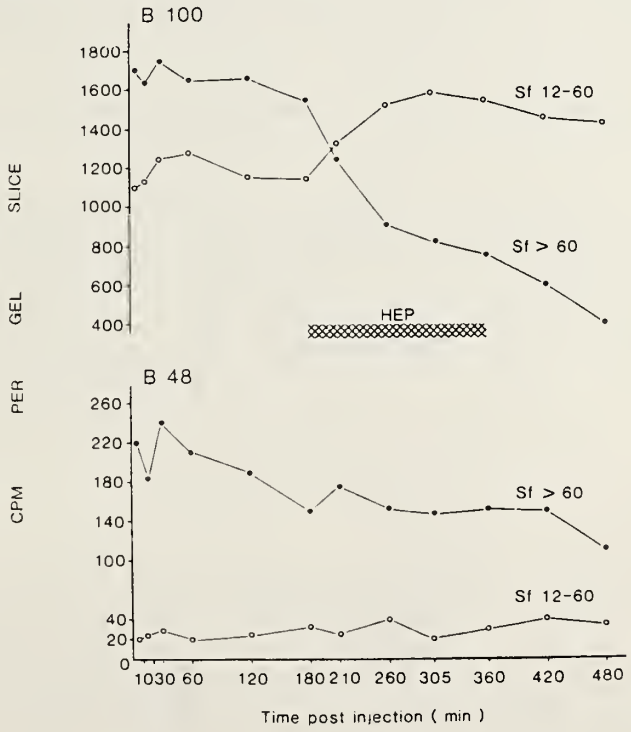

Figure 1. Changes in apo B100 and apo B48 radioactivity concentrations in Sf>60 and Sf12-60 lipoproteins after reinjecting ^{125}I-TRL; effect of heparin infusion in a Type 5 hyperlipoproteinemic subject.

Figure 1 summarizes the results in one subject, which typifies the overall findings. In all 4 subjects, the decline in both the apo B48 and apo B100 radioactivity curves was initially very slow. Heparin induced a clear increase in the disappearance of apo B100 radioactivity from Sf>60 in all 4 subjects. This was not the case for apo B48: only in one subject was there some response which was however less than for apo B100 (Table 1). Furthermore transfer of apo B48 radioactivity from Sf>60 to the Sf12-60 lipoproteins was not accelerated with heparin which was a marked feature of apo B100 metabolism. Post-heparin lipolytic activity comprising both hepatic and extrahepatic lipases, was present in all subjects.

Table 1 Heparin induced removal of TRL

Subject	Increase in apo B removal (%)	
	B48	B100
1	0	27
2	0	33
3	0	51
4	37	55

TRL = Sf>60
Constant infusion of heparin over 3 hr

Thus, increased plasma lipolytic activity accelerated chylomicron (remnant) removal very much less than VLDL (remnant) removal in Type 5 hyperlipoproteinemic subjects. This assumes that apo B48 and apo B100 clearly differentiate between the two classes of remnants. It suggests that a major defect leading to Type 5 hyperlipoproteinemia is failure of chylomicrons to become degraded to the point where the particles can interact optimally with the hepatic chylomicron remnant receptor. The alternative interpretation that of defective chylomicron receptor activity in this disorder, is a less appealing possibility.

TRL, including chylomicron remnants, can be removed by tissues other than liver. In particular, macrophages have been reported to bind, internalize and degrade large TRL obtained from plasma following fatty meals.[10-12] Incubation of TRL, which include chylomicron remnants, with macrophages of several kinds, leads to massive overload of the cells with triglyceride. Cholesterol increments are modest so that the resultant foam cell does not resemble the cholesterol laden cells of atheroma such as is induced with incubations of β-VLDL or chemically modified LDL. Nevertheless, the substantial uptake of TRL by macrophages shares some of the characteristics of β-VLDL uptake and differs from the very minor uptake of normal VLDL.

The large capacity of macrophages to catabolize TRL may have added significance in Type 5 hyperlipoproteinemia, where the normal removal mechanisms are inefficient and the plasma levels of chylomicrons and their remnants are high. We have therefore examined the nature of the interaction between the largest TRL circulating in such patients and two macrophage lines, the rat peritoneal macrophage and the J774 transformed mouse macrophage.[11] The findings with the latter are presented here.

Chylomicron remnants were isolated from fasting subjects with Type 5 hyperlipoproteinemia as the Sf>60 lipoprotein fraction. The largest particles were further isolated on 4% agarose columns. These particles termed TRL_1 differed from smaller TRL (TRL_2) by having a higher content of apo B48, and of triglyceride and also a higher content of apo E relative to apo C. TRL_1 were metabolized by macrophages to a greater extent than TRL_2. Further, as shown in Figures 2 and 3 (typical of each of 6 different samples), TRL_1 competitively displaced ^{125}I-β-VLDL as effectively as β-VLDL itself. TRL_2 was less effective and VLDL obtained from hypertriglyceridemic (Type 4) subjects, least. Thus, chylomicron remnants from patients with Type 5 hyperlipoproteinemia were removed by macrophages more readily than were VLDL, suggesting a different removal mechanism from that operating in vivo in the liver.

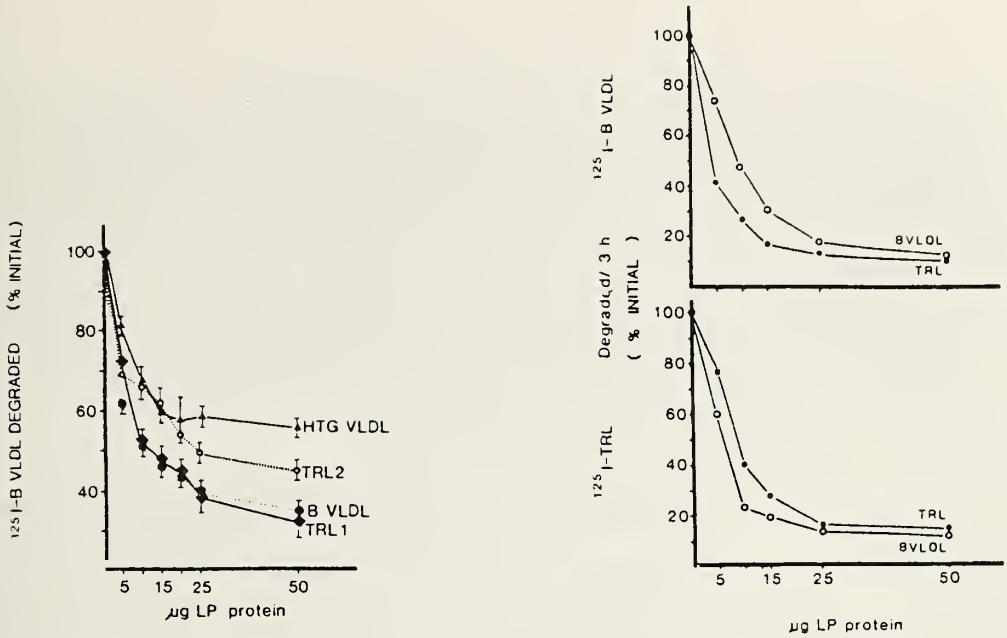

Figure 2 and 3. Uptake of TRL at sites that also recognize β-VLDL. Displacement by TRL_1, TRL_2 and hypertriglyceridemic VLDL of ^{125}I-β-VLDL in fresh rat peritoneal macrophages (Fig. 2, left). Mutual displacement of TRL and of β-VLDL in J774 macrophages (Fig. 3, right).

Since β-VLDL can bind to macrophages through a variety of ligand-receptor interactions (a postulated β-VLDL receptor, the LDL receptor and an apo E/chylomicron receptor-like site), we carried out further experiments which would normally suppress the LDL receptor. Preincubating the cells with fetal calf serum which suppressed LDL uptake, did not influence the capacity of TRL to displace β-VLDL (Figure 4). That the LDL receptor was not involved in TRL catabolism by macrophages was further demonstrated by the failure of monoclonal antibodies raised against apo B48 but reacting also with apo B100, to inhibit TRL uptake (Figure 5).

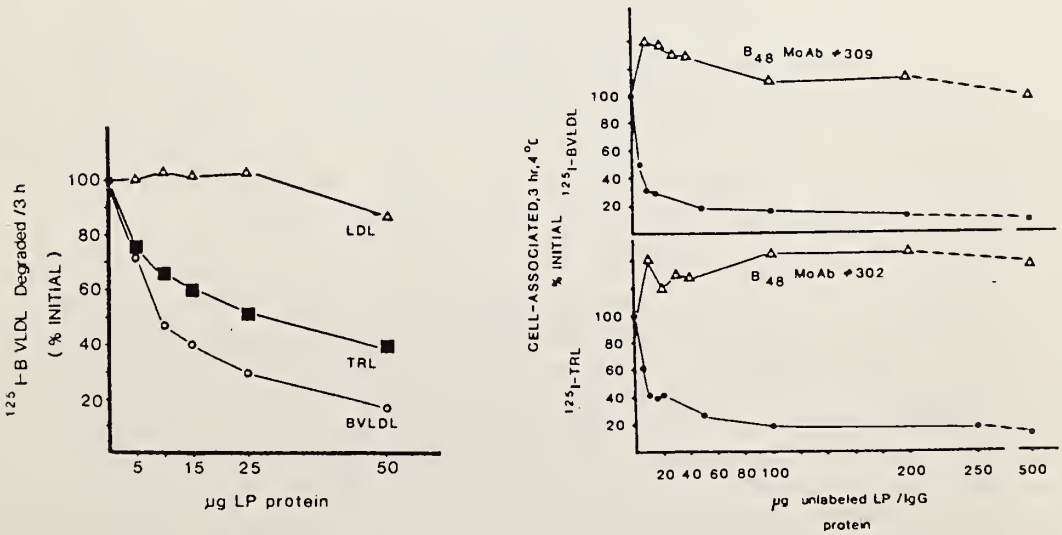

Figure 4 and 5. Independence of TRL and LDL uptake. Displacement of ^{125}I-β-VLDL by LDL and TRL following suppression of LDL receptor by (a) preincubation of cells with fetal calf serum (Fig. 4, left) or (b) preincubation of ^{125}I-TRL or ^{125}I-β-VLDL with monoclonal antibodies against B48 and B100 (Fig. 5, right).

The higher apo E/C ratio in TRL_1 than in TRL_2, was a possible reason for the greater uptake of TRL_1 by the macrophages. Apo E has been shown to be necessary for the catabolism of chylomicron remnants from normal subjects in human monocyte-macrophages[10] and we had shown that the addition of apo C to rabbit β-VLDL suppressed their uptake by peritoneal macrophages.[11] We therefore separated TRL from Type 5 patients by another technique, heparin-affinity chromatography. The particles that bound to the column showed 6-fold greater apo E/C ratios than particles which eluted and were unbound. However, despite these large differences both classes of particles increased the cholesterol content of J774 macrophages by similar amounts (ca 150%).[13]

Table 2 Increments in Cellular Cholesterol

Lipoprotein 100 μg/ml (n=5)	Cell Cholesterol μg/mg protein
No lipoprotein	19± 3
ApoE-rich TRL*	50± 7
ApoE-poor TRL*	46± 3
Normal VLDL	29± 3
Normal LDL	26± 1

Huff M, Nestel PJ 1986
*Separated by Heparin-Sepharose

On the other hand, the nature of the apo E was important. TRL obtained from patients with Type 3 hyperlipoproteinemia 3 hours after a fatty meal failed to raise the cholesterol content by more than 20%. Type 3 hyperlipoproteinemia is characterized by apo E_2 which does not interact with apo E dependent receptors including the chylomicron receptor. This finding does suggest a role for normal apo E (apo E_3 or apo E_4) in the uptake of chylomicron remnants by macrophages. Our finding does not conflict with the classic observations[14] that β-VLDL from subjects with Type 3 hyperlipoproteinemia induce massive cholesterol overload in some species of macrophages. Macrophages in which cholesterol overload has been demonstrated have the capacity to secrete apo E into the medium which presumably then associates with the apo E_3 deficient β-VLDL. We chose the J774 line because it lacks the capacity to form and secrete apo E. However, if β-VLDL uptake by macrophages is independent of apo E_3, yet TRL uptake is, then TRL may not be taken up through the putative β-VLDL receptor.

We conclude that particles enriched in chylomicrons and remnants, from Type 5 hyperlipoproteinemic subjects, are avidly taken up by macrophages at sites that also recognize β-VLDL. Whereas normal apo E_3 may be a requirement, there is not a clear relationship between apo E content and TRL catabolism. Three other very recent studies bear on this. Gianturco et al.,[12] using P388D macrophages also concluded that chylomicron remnants are taken up at β-VLDL binding sites but that apo E may not be the obligatory ligand. Bersot et al.[15] have suggested that Lp(a) may be involved. Van Lenten et al.[10] have postulated that chylomicron remnants are recognized by a macrophage receptor specific for the remnant, in addition to other recognition sites such as the β-VLDL and possibly the LDL receptor. Whereas the latter two receptors could be down-regulated by overloading the human monocyte macrophages with sterol, the low-affinity high-capacity chylomicron remnant receptor remained unaffected. Apo E appeared to be the preferred ligand.

Our in vivo and in vitro studies of TRL metabolism, taken together, suggest that the binding site for chylomicron remnants on macrophages is not the receptor present in the liver. TRL which in vivo were resistant to normal removal were taken up at an enhanced rate in vitro. It should however be noted that J774 macrophages can secrete lipoprotein lipase, so that the enhanced in vitro uptake of TRL might have reflected preferential uptake of the apo B100 particles, as observed in vivo. This is unlikely since TRL, which included a substantially higher proportion of apo B48 particles, was metabolized by macrophages at a greater rate than TRL_2, which comprised mainly apo B100 particles.

Furthermore, lipolysis of human lymph chylomicrons does not appear to be a prerequisite for their uptake by macrophages.[16]

Our studies also exclude a role for the conventional LDL receptor in the in vitro removal of the TRL.

The macrophage system may partly compensate in vivo for the otherwise ineffective removal of chylomicron remnants in Type 5 hyperlipoproteinemia. This alternate avenue for TRL clearance does not apparently require the degree of delipidation of the particle which is necessary for binding to the chylomicron remnant receptor. Whether this system contributes significantly to atherosclerosis is not clear. This study as well as that of Van Lenten et al.[10] establish that a modest cholesterol loading of macrophages is possible through the uptake of chylomicron remnants. This may reflect the much greater capacity of these cells to mobilize, through lipolysis, the triglyceride than the cholesterol load.[16] Chylomicrons have also been shown to bind to vascular endothelial cells and deliver both cholesteryl ester and triglyceride into the cell.[17] Atherosclerosis is however not a marked feature of Type 5 hyperlipoproteinemia.

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SESSION II. DISCUSSION OF HETEROGENEITY OF TRIGLYCERIDE-RICH LIPOPROTEINS ASSESSED IN VITRO

DR. HAVEL: Dr. Gianturco, I found the last part of your presentation particularly fascinating with regard to the protein content of particles and relationships and accessibility to the antibody and also the receptor binding properties. As you recall, our laboratory has been emphasizing for a number of years the importance of C proteins in the metabolism of both chylomicrons and VLDL remnants. I propose it's the loss of C proteins from its recognition by the chylomicron receptor as well as the LDL receptor and ApoE contained in these remnant particles. It was shown also by Windler a number of years ago, in collaboration with the Brown and Goldstein laboratory, that addition of C proteins to VLDL particles that are well-recognized by the LDL receptor can, without loss of any E on the particle, totally prevent that recognition. So that is all quite consistent. I mention this partially just to remind people that there is other data that relates to this, but also to ask Dr. Gianturco some questions that relate to the stoichiometry because it has always puzzled me why a large VLDL particle that has only one molecule of ApoE in an accessible conformation and has an affinity for the LDL receptor that is similar to LDL could have completely different metabolic properties. LDLs circulate for days. Those large VLDLs circulate for minutes and are not converted to LDL normally. You have now shown that some VLDL may have more accessible E's, but the bulk of those that you published had only one. I wonder whether those are the particles that are being metabolized by receptors, or if the ApoC is first lost during lipolysis and at the moment that it is lost the particle has a residence time in plasma that may be 30 seconds and therefore you never see it.

DR. GIANTURCO: Clearly, it is puzzling that the VLDL residence time is somewhat less, but I do want to point out that I believe the residence time of VLDL in moderate hypertriglyceridemic subjects is more like six hours as opposed to minutes. So that does give a reasonable amount of time for these two to be taken up. Some of the studies that Dr. Packard is going to present later on will indicate that it can be taken up in vivo.

Normally, the case is as you say. They are lipolized to the $VLDL_3$ size before they are taken up. Of course, if E is there, it could mediate binding through either the E receptor or the LDL receptor. We found in vitro that E is not necessary for binding at least small particles to the LDL receptor. Dr. Krul also showed that the B is in the appropriate conformation there and the antibody against B blocks binding, but the antibody against E does not. As you pointed out, the stoichiometry of E, in our initial studies in which we first published the thrombin accessibility were done with subjects who were moderately hypertriglyceridemic and on the average had one to two moles of accessible ApoE. We've seen many other subjects who have more accessible E than that. The K_D goes down dramatically the more E that is on the particle but it reaches a limiting value of about half nanomolar K_D no matter how many ApoE's are present. We've found up to 16 moles of E from a type three subject and it has a K_D of half nanomolar and you can get the same results with six moles of E per mole of VLDL-1. I'm not exactly sure what this means but I don't think it is multiple receptor binding. I think it just means that there are more ApoE's on the surface of VLDL at the appropriate conformations so that no matter how that large VLDL approaches the receptor, the more Es on there, the greater because each collision has a better chance of landing on the right one. So, probably it is just increasing statistical chances of binding appropriately.

DR. HAVEL: What I'm suggesting is that the numbers that are accessible in a plasma sample may not reflect the number that are accessible at the moment that binding occurs which may be mediated, among other things, by loss of C proteins.

DR. GIANTURCO: Oh, yes. That very probably has a major influence.

DR. FISHER: Dr. Gianturco, a little physical chemistry, that may be useful. If you isolate from hypertriglyceridemia VLDL, the 1006 to 1019 fraction, there are several species certainly of lipoproteins, but the predominant one is an S_f20 lipoprotein. When one does the hydrodynamic studies on this, the hydrated density on this particle is 1004. Now, what this means is that when you do a centrifugation of 1006, you will distribute your major IDL throughout the tube. When you then take off the supernatant, VLDL, you will remove a fair bit of IDL. When you bring the density of the infranatant, up to 1019, you will spin up the rest of your IDL. So, very clearly, in terms of physical properties, there has got to be a considerable amount of what we refer to normally as IDL in the VLDL.

DR. GIANTURCO: Oh, absolutely, yes. I showed in one of the slides that we found a great deal of IDL in the VLDL fraction. In a normal subject, if you do a single 18-hour spin and take the traditional 1006 top or VLDL, we find up to 50 percent of the protein present in the 1006 top is IDL when we then analyze it by ultracentrifugation.

DR. FISHER: The physical properties of protein necessitate that.

SPEAKER: Dr. Gianturco when you discussed possible ways in which hypertriglyceridemic VLDL might be atherogenic, it appears that you left out one possibility. Couldn't you simply also speculate that when there is hypertriglyceridemic VLDL in the circulation that will go into the cells, including the liver cells, via the LDL receptors and cause the down-regulation of LDL receptors?

DR. GIANTURCO: That's right. That's very important. That is one of the things that happens. You do get the down-regulation.

DR. GINSBERG: Dr. Nestel, do you have an S_f distribution of the S_f greater than 60 particles, a B-48 distribution across the S_f range and the B-100 distribution.?

DR. NESTEL: Yes, we've looked at 100 to 400 as well as sixty to 100 and greater than 400. There is substantial B-48 right down to the S_f-60 range, but, as you indicate or imply, the ApoB-48 rich particles are present in greater amounts in the less dense particle range. Although the average particle size wasn't all that much different, the TRL1 were about 130 nanometers and the TRL2 were about 110.

DR. GINSBERG: Dr. Nestel, the results of the heparin infusion are somewhat inconsistent with that. Your hypothesis is that the B-48 particles never reach a remnant stage and yet, if there is a significant proportion of B-48 bound in the lower S_f range, I wouldn't expect them to be removable at that point.

DR. NESTEL: That's not entirely my hypothesis. Most of the things that we see in the plasma are probably chylomicron remnants. All I'm saying is that they haven't yet,for one reason or another, reached the degree of delipidation where the other conformation of the ApoE or the presence of the ApoC is such or the absence of ApoC has reached a stage where binding can occur.

DR. WITZTUM: I would like to remind everyone of a paper that was published about 20 years ago from Seattle. Palmer and Bierman were the authors. They looked at serial biopsies in the skin of patients with type five hypertriglyceridemia during the period of the marked hyperlipemia and then serially as the hyperlipemia went away. They pointed out that the lipids that were found in these lesions, predominantly in foam cells, were initially triglyceride-enriched, presumably reflecting uptake in these macrophages in the skin of the hypertriglyceridemic chylomicron lipoproteins. But, as the hypertriglyceridemia regressed, the lesion composition changed so that there was a much greater and more rapid mobilization of triglyceride from the macrophage leaving behind a relative cholesterol enrichment. As John Koon has subsequently shown in San Diego it is a very active neutral triglyceride lipase. Another in vivo and very important process that could lead to the net accumulation of cholesteryl ester from chylomicron remnants is the fact that you mobilize out relatively the triglyceride. One could imagine that in a steady state situation where there is constant uptake and mobilization of chylomicron remnants, every time a chylomicron remnant is internalized in a macrophage, there will be a net accumulation of cholesteryl ester over time.

DR. PACKARD: Dr. Nestel, interpretation of your heparin experiments would probably be that both events--the formation of the remnant would occur at S_f greater than 60. In type threes and also in type fives there are both alpha-migrating and beta-migrating VLDL in the large VLDL range. So, it is possible that during heparin infusion big chylomicrons are transformed into chylomicron remnants, but they never get any smaller than S_f greater than 60. Then they are removed by the receptors processes. We've done turnover studies with native and cyclohexanedione treated VLDL in types fives at S_f 100 to 400 and 60 to 100. About half of the lipoprotein triglyceride seems to be removed by a process that depends on arginines, a process that is receptor dependent. So, probably all of the processes are moved up into that chylomicron range and that's why you are not seeing the decrease.

DR. NESTEL: Not entirely, Dr. Packard. That had occurred to us. You are saying that there is a redistribution within the S_f range that we've measured. That's why I replied to Dr. Ginsberg and said we did look at the subfractions also; S_f greater than 100 to 400, greater than 400, and the S_f 60 to 100, but I only summarized the one lot here. What you're saying isn't entirely correct in that we were not able to mobilize substantial amounts of B-48 activity from greater than 100 into the 60 to 100. We were able to mobilize material from the 60 to 100. We were able to mobilize material from the greater than 400 into the 100 to 400. But, in any case, there should have been a net effect in the B-48 radioactivity disappearance one way or the other. Even if one has a transfer, there must be a loss. If there is no net loss, then there isn't the same degree of uptake as there is with the B-100 fraction.

SPEAKER: Dr. Nestel, have you looked at the specificity of the C-III inhibition of TRL2? Will the C-I or C-II also inhibit it in the same degree as your C-III does? Is there any difference between C-III, C-I, and C-II?

DR. NESTEL: C-II inhibited also, but at about half the extent as the CIII-1 and CIII-2.

SPEAKER: Does C-I work at all?

DR. NESTEL: We didn't have enough.

DR. GINSBERG: Dr. Krul, have you looked at the profile or the thiocyanate-eluted material and collected small fractions and looked at the distribution of apoproteins,

lipids, and particularly in your case ApoE? We found with three mole of thiocyanate the VLDL particles come off the anti-E column, but that the E is torn off the particle, especially when you have a G-25 column layered below you would gel chromatograph your VLDL away from the free E. If you collect a large volume, it seems to us that it reassociates appropriately, but, if you are collecting small fractions and only using some volume of elution, that you might make a new type of particle. I wondered if you'd looked at that?

DR. KRUL: No, we haven't looked at that, but we do pool all of the fractions. We do collect it as a batch. We establish the conditions by using small fractions to see where exactly the elution absorbant goes back down. We always pool the fractions together. We do have very little thiocyanate in the particle. I don't know how much time is required for the thiocyanate to react, but basically the column elutes in about ten minutes. The thiocyanate is probably in the presence of lipoprotein only for ten minutes. So, I'm hoping, since I am concerned with the ApoE configuration, that nothing happens to the particle. We'd like to do more extensive studies and some EM work and more critical lipid analyses. Maybe what you suggest would be a good idea, to see if that in fact is happening. We would like to see whether the particles that we're pulling off, are homogeneous or still a heterogeneous population of particles.

SESSION III. Heterogeneity of Cholesteryl Ester Rich Lipoproteins Assess *In Vitro*

Chair: Alan Fogelman
Cochair: Paul Nestel

HDL-WITH APOLIPOPROTEIN E: IMPORTANT CONSIDERATIONS AND METABOLIC SIGNIFICANCE OF THIS HIGH DENSITY LIPOPROTEIN SUBCLASS

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Introduction

Epidemiological evidence has demonstrated that lower plasma levels of high density lipoproteins (HDL) correlate with a higher incidence of coronary heart disease (1-3). These observations suggest that HDL may be antiatherogenic and have stimulated investigation into how they might have this effect. Because HDL are heterogeneous in size, density, and chemical composition, it is essential to understand this heterogeneity in terms of metabolic behavior. This review will focus on one of the metabolically distinct subclasses of HDL, the HDL-with apolipoprotein (apo-) E. Consideration will be given to the proposed role of this lipoprotein subclass in two potentially antiatherogenic mechanisms: reverse cholesterol transport and, perhaps more importantly, the redistribution of cholesterol among cells (4, 5). In addition, the influence and effects that the various apo-E isoforms have on human HDL-with apo-E will be considered.

Canine Plasma HDL

Because the HDL-with apo-E subclass has been best characterized in the dog (6), the information from this model can serve as a foundation when considering other species. As diagrammed in Figure 1, dog plasma contains a heterogeneous spectrum of HDL particles. (A similar HDL spectrum exists in rat plasma [7]). The various canine plasma HDL subclasses can be conveniently isolated by a combination of ultracentrifugation and Geon-Pevikon block electrophoresis (6). Above a density of 1.087 g/ml, small (70- to 100-Å diameter) apo-A-I-rich particles that are equivalent to human HDL_2/HDL_3 subclasses are present. Below d = 1.087 g/ml, apo-E is associated with HDL particles. The larger and less dense the particle, the more apo-E there is. The lipoproteins in the d = 1.02-1.087 range are referred to as HDL_1 and fall into two size ranges, the 140- to 160-Å and the 180- to 200-Å diameter particles. At the end of this spectrum, in the 1.006 to 1.02 density range, are the apo-E HDL_c: apo-E is their sole apolipoprotein component. The apo-E HDL_c, with a half-life of approximately 20 min, are rapidly removed from the plasma of dogs and rats. The liver is the major site of uptake, and hepatocytes are responsible for their rapid removal (8). Thus, the apo-E HDL_c are effectively targeted to the liver for their clearance.

In the dog, as well as in other animals, a high-cholesterol and high-fat diet raises the concentration of the HDL-with apo-E subclasses in the plasma (for review, see reference 9). A similar increase occurs with egg feeding in humans, although the increase is not as dramatic (10). Species in which the HDL-with apo-E are prominent (dogs, rats, swine) are characterized by low levels of cholesteryl ester transfer activity (11). Presumably, low activity results in the accumulation of HDL-with apo-E in

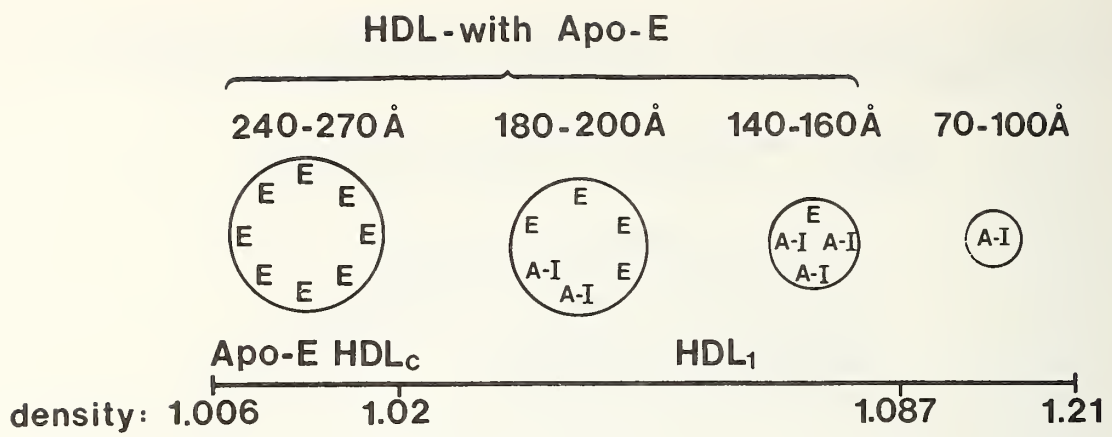

Figure 1. Spectrum of canine plasma HDL particles.

reverse cholesterol transport. This point will be considered in more detail in the section on the metabolic role of HDL-with apo-E.

Two important general characteristics of apo-E-containing lipoproteins can be illustrated with canine apo-E HDL_c. First, because apo-E has the ability to bind to apo-B,E(LDL) receptors, apo-E HDL_c are capable of delivering cholesterol to cells expressing these receptors (12). Second, apo-E HDL_c bind with a higher affinity to apo-B,E(LDL) receptors than low density lipoproteins (LDL) and therefore are more effective competitors for receptor binding sites (13). This enhanced binding of apo-E HDL_c — approximately 10- to 20-fold — is the result of two factors: 14 to 16 copies of apo-E are present on an apo-E HDL_c particle, and multiple ligand binding sites are present on a single receptor molecule (14). Thus, through multiple interactions with a single receptor, up to four apo-E molecules on an apo-E HDL_c particle can participate in the binding. In contrast, LDL appear to have a single binding site per particle and can only interact with a single ligand binding site on the receptor (13).

Human HDL-with Apo-E

Although fractions enriched in HDL-with apo-E can be obtained from the d = 1.063-1.125 g/ml range of human plasma by ultracentrifugation (15), a more reproducible method employs heparin-Sepharose affinity chromatography. The method takes advantage of the fact that apo-E binds with a moderately high affinity to heparin. A gradient salt elution can resolve the d = 1.063-1.21 fraction into three subfractions: 1) nonbound HDL, which does not contain apo-E, 2) HDL-with apo-E, and 3) a β-migrating apo-B-containing fraction (16). The HDL-with apo-E subclass represents approximately 5 to 6% of the apolipoprotein mass present in the 1.063 to 1.21 density range. The HDL-with apo-E have larger particle diameters, as determined by negative staining electron microscopy, than the HDL-without apo-E (100 to 150 Å versus 80 to 120 Å).

Polyacrylamide gel electrophoresis of the three heparin-Sepharose subclasses demonstrated that the nonbound HDL does not contain apo-E (Fig. 2). The majority of apo-E in HDL-with apo-E was present as the M_r = 45,000 disulfide heterodimer apo(E—A-II). Disulfide-reducing agents converted the apo(E—A-II) to the apo-E and apo-A-II subunits. The HDL-with apo-E isolated by the heparin-Sepharose method competed effectively with LDL for apo-B,E(LDL) receptors on cultured fibroblasts (16), whereas the HDL-without apo-E did not. Thus, based on the presence or absence of apo-E, total HDL were subfractionated into two metabolically distinct subclasses by heparin-Sepharose affinity chromatography.

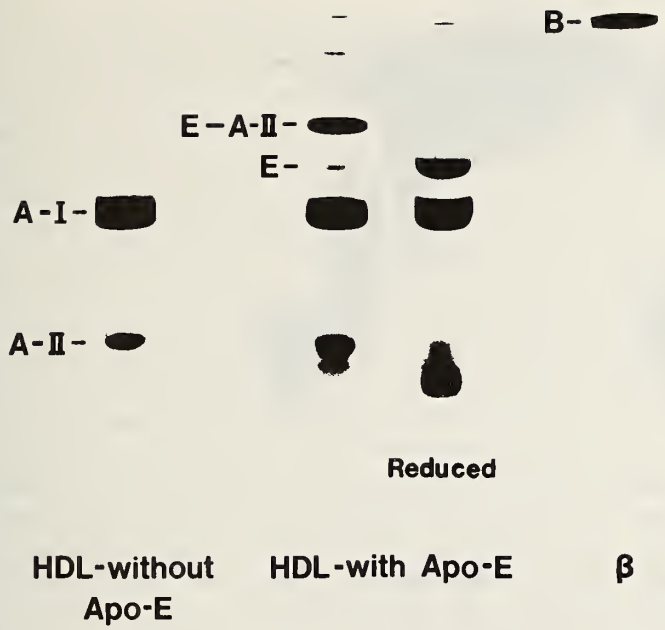

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the heparin-Sepharose subclasses isolated from the d = 1.063-1.21 g/ml fraction of human plasma.

Because apo-E is stripped from lipoprotein particles during ultracentrifugation (17), an alternative method to isolate HDL-with apo-E was considered. Previously, we have demonstrated that canine apo-E HDL_c can be isolated directly from plasma without ultracentrifugation by using a combination of agarose column chromatography and Geon-Pevikon block electrophoresis (8). The method chosen for the isolation of human HDL-with apo-E was a combination of agarose chromatography and anti-apo-E immunoaffinity chromatography (18).

Plasma from a patient with type III hyperlipoproteinemia was used because of its elevated levels of apo-E. Fractionation of the plasma resulted in an elution profile similar to those reported for plasma from normal subjects (18). The apo-E-containing HDL elute before the bulk of the HDL, demonstrating that the HDL-with apo-E are larger than the apo-A-I-rich HDL (18). These results are consistent with those obtained by the heparin-Sepharose method (16). The apo-E-containing HDL eluted in a broad peak that was divided into two fractions. As will be described below, each fraction contained a different size range of HDL-with apo-E particles. Each fraction was immunopurified with anti-apo-E immunoaffinity columns. As determined by polyacrylamide gel electrophoresis of the immunopurified fractions (data not shown), both apo-E and apo-A-I were present. The patterns resembled those of the HDL-with apo-E separated by heparin-Sepharose chromatography (Fig. 2).

To examine the size heterogeneity of these apo-E-containing lipoproteins in plasma and those fractions isolated by agarose chromatography, an immunoblotting technique employing 4-30% gradient polyacrylamide gel electrophoresis was developed (Fig. 3). After their transfer to nitrocellulose filters, the apo-E-containing lipoproteins were detected by using ^{125}I-labeled anti-apo-E immunoglobulin G and autoradiography. This direct detection method was necessary because the commonly used secondary detection reagents (iodinated Protein A or anti-rabbit immunoglobulin G) would also detect immunoglobulins present in unfractionated human plasma. As indicated in Figure 3, there is size heterogeneity in the lower portion of the gel, where HDL-sized particles migrate. This range of sizes is similar to that observed for dog plasma (19), indicating that human plasma contains HDL-with apo-E particles similar in size to those in Figure 1. This is consistent with previous studies, which suggested that small human HDL particles expand as they acquire cholesterol, producing a broad size

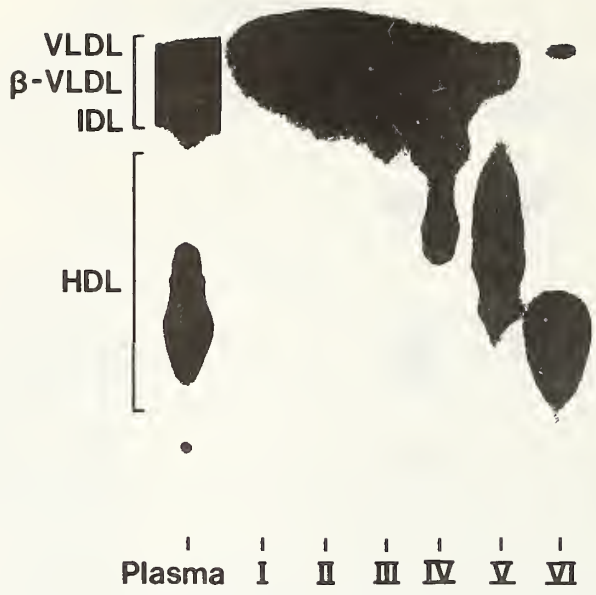

Figure 3. Immunoblot of 4-30% gradient polyacrylamide gels of type III hyperlipoproteinemic plasma and agarose column fractions. The plasma and the six fractions across the Bio-Gel A5M column profile of type III hyperlipoproteinemic plasma (lanes I through VI) were electrophoresed on 4-30% gradient gels and transferred to nitrocellulose filters. The apo-E-containing lipoproteins were detected with ^{125}I-labeled affinity-purified anti-apo-E immunoglobulin G.

range of HDL-with apo-E particles (19). After agarose column chromatography of type III hyperlipoproteinemic plasma, the majority of the HDL-with apo-E particles eluted in Fractions V and VI. Fraction V contained larger particles than Fraction VI, as illustrated on the gradient gel (Fig. 3). The gel makes it possible to distinguish at least three or four different apo-E-containing HDL subclasses. The apo-E-containing very low density lipoproteins (VLDL), β-VLDL, and intermediate density lipoproteins (IDL) are present in Fractions I through IV. When plasma from normal individuals was examined, a similar size heterogeneity of HDL was also observed (data not shown).

Apo-E Isoforms and HDL-with Apo-E. It has been demonstrated that the three most common apo-E isoforms differ in their cysteine content: apo-E2 contains two cysteines (residues 112 and 158), apo-E3 contains one cysteine (residue 112), and apo-E4 lacks cysteine (20, 21). As a result of these differences, apo-E2 and apo-E3 can form the disulfide heterodimer apo(E—A-II), but apo-E4 cannot. This is important when considering HDL-with apo-E because, as Figure 2 shows, most of the apo-E is complexed with A-II (15). This general property of HDL-with apo-E in subjects who have the cysteine-containing apo-E2 and apo-E3 is significant: when apo-E is complexed with apo-A-II on a HDL particle, it does not bind to apo-B,E(LDL) receptors as effectively as uncomplexed apo-E (22). The binding activity of the HDL-with apo-E particles is substantially increased after reduction of the apo(E—A-II). Therefore, it has been suggested that apo(E—A-II) might serve to modulate the binding activity of HDL-with apo-E *in vivo* (22).

The apo-E4/4 phenotype is characterized by several interesting

properties; compared with other phenotypes, for example, a higher percentage of plasma apo-E in E4/4 subjects is associated with VLDL (23). Individuals possessing the apo-E4 isoform tend to have higher plasma cholesterol and LDL levels than those possessing apo-E2 or apo-E3 (24-26). In addition, the plasma apo-E levels are lower in apo-E4/4 subjects. Apolipoprotein E4 has these characteristics even though it appears to bind normally to apo-B,E(LDL) (27) and apo-E receptors (unpublished data).

Could these unusual characteristics of the apo-E4/4 phenotype be accounted for by the inability of apo-E4 to form apo(E—A-II)? Let us first consider the abnormal plasma distribution of apo-E. In research on E4/4 and E3/3 heterozygotes, it was demonstrated that the apo-E4 isoform is preferentially distributed in VLDL (23). Our approach to examine apo-E isoform distribution was to determine whether apo-E4 and E3 were distributed differently in the same subject, i.e., an apo-E4/3 heterozygote. Plasma from a subject with the E4/3 phenotype was fractionated into VLDL and HDL, and the isoform distribution was examined by isoelectric focusing and immunoblotting. As shown in Figure 4, the amount of apo-E4 in VLDL is much greater than in HDL. In addition, the majority of the apo-E3 in the HDL existed as apo(E—A-II) (data not shown). Although some apo(E—A-II) is present in VLDL, the major portion is found in HDL. These results suggest that the formation of apo(E—A-II) might play a direct role in the differential plasma distribution of apo-E4 and E3 in VLDL and HDL.

It is also possible that apo-E4's inability to form a disulfide complex with apo-A-II explains some of the other characteristics associated with the apo-E4/4 phenotype, namely, high LDL levels and low apo-E levels in the plasma. Because apo-E4 cannot complex with apo-A-II to form apo(E—A-II), apo-E4 is in a receptor-active form and is preferentially distributed in VLDL. The VLDL would be removed from circulation by cells (primarily hepatocytes) expressing apo-B,E(LDL) receptors, with several consequences: The VLDL uptake would lower the plasma level of apo-E4; the uptake of VLDL cholesterol would result in down-regulation of hepatic apo-B,E(LDL) receptors; and because there are fewer receptors expressed, plasma LDL levels would rise.

Another consideration relevant to this discussion is the receptor binding activity of these various apo-E isoforms. The structures of several apo-E variants have been determined, and their receptor binding properties have been examined using purified protein recombined with the phospholipid dimyristoylphosphatidylcholine (DMPC). Compared with apo-E3 and apo-E4($Cys_{112} \rightarrow Arg$), which display normal binding activity, several variants are defective in their ability to bind to the apo-B,E(LDL) receptor (for review, see reference 28). Consideration was given to the possibility that apo-E on an HDL particle might not display the same receptor binding characteristics that it does on a DMPC recombinant. To address this point, HDL-with apo-E were isolated by heparin-Sepharose

Figure 4. Immunoblots of isoelectric focusing gels of VLDL and HDL fractions obtained by fractionating plasma from a subject with an apo-E4/3 phenotype on an agarose (A5M) column. After isoelectric focusing and transfer to nitrocellulose filters, the apo-E isoforms were detected with ^{125}I-labeled affinity-purified anti-apo-E immunoglobulin G.

chromatography from a control apo-E3/3 subject and from two subjects with apo-E2 variants. The HDL-with apo-E were reduced and alkylated to release the apo-E complexed with apo-A-II, and the apo-E content was determined by radioimmunoassay. The first apo-E2/2 subject was homozygous for E2(Arg_{158}→Cys), and the second was a heterozygote possessing E2(Arg_{158}→Cys) and E2(Arg_{145}→Cys). As shown in Figure 5, the HDL-with apo-E from the subject homozygous for E2(Arg_{158}→Cys) displayed the lowest binding activity. The HDL-with apo-E from the E2(Arg_{158}→Cys)/E2(Arg_{145}→Cys) heterozygote possessed an intermediate binding activity, although it was defective relative to the normal E3/3 control.

To make a direct comparison between receptor binding results of the HDL-with apo-E and the DMPC recombinant, the apo-E concentration at which 50% of the ^{125}I-LDL was displaced from the receptors was determined for each sample. The results from each series were then normalized to their own apo-E3/3 control, which was set at 100% binding activity. As Table 1 shows, apo-E on an HDL particle behaves almost exactly as it does when complexed with DMPC. Thus, the receptor binding defect displayed by several apo-E variants will manifest itself in HDL-with apo-E.

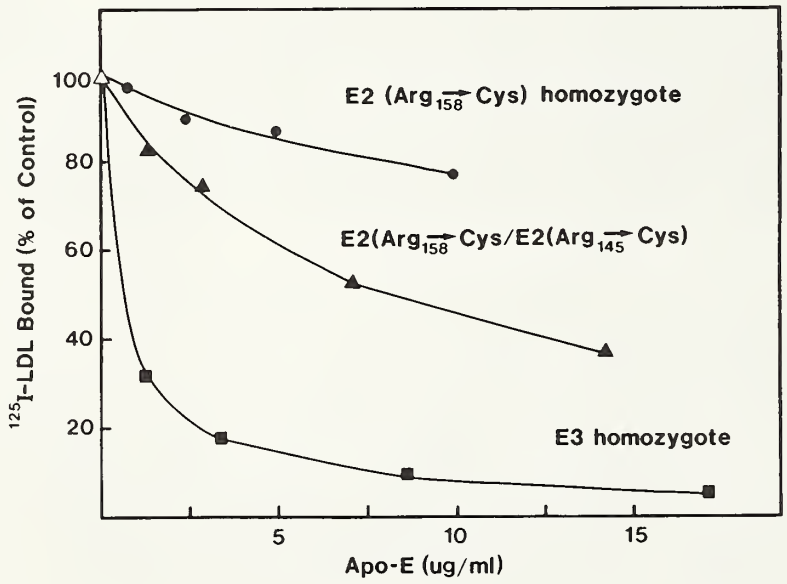

Figure 5. Ability of various reduced and alkylated HDL-with apo-E to compete with ^{125}I-LDL for binding to the apo-B,E(LDL) receptor on human fibroblasts. The HDL-with apo-E were isolated from the d = 1.063-1.21 g/ml fraction of human plasma by heparin-Sepharose affinity chromatography and reduced and alkylated by treatment with β-mercaptoethanol and iodoacetic acid. Increasing concentrations of HDL-with apo-E were added with 2 μg of ^{125}I-LDL/ml in Dulbecco's modified Eagle's medium containing 10% lipoprotein-deficient serum. The apo-E content was determined by solid-phase radioimmunoassay. After a 2-hr incubation at 4 C the cells were extensively washed with cold phosphate-buffered saline containing 2 mg of bovine serum albumin/ml, dissolved in 0.1 M NaOH, and counted for radioactivity.

Table I. Comparison of receptor binding activities of apo-E.DMPC complexes and HDL-with apo-E.

Genotype	Binding Activity	
	Apo-E.DMPC	HDL-with apo-E
E3 homozygote	100%	100%
E2(Arg_{158}→Cys)/E2(Arg_{145}→Cys)	7%	6%
E2(Arg_{158}→Cys) homozygote	1%	1%

Metabolic Role of HDL-with Apo-E

The increase in the plasma concentration of HDL-with apo-E in cholesterol-fed animals suggested that this lipoprotein subclass plays an important role in cholesterol transport. It has also been suggested that this subclass might be a component of the reverse cholesterol transport process and could therefore be potentially antiatherogenic (4, 5, 9). To explore this possibility, a series of experiments designed to examine the potential role of HDL-with apo-E in the reverse cholesterol transport process was conducted (19, 29). These studies showed that small HDL_2/HDL_3 particles could be converted into larger particles by using canine plasma and an external source of cholesterol. Similar results were obtained by using human plasma (19). The results of these studies are summarized in the model shown in Figure 6.

Further in vitro incubation studies (29) demonstrated that small particles of HDL-without apo-E could be converted into a spectrum of larger apo-E-containing HDL particles similar to those shown in Figure 1, provided three conditions were met. First, an external source of cholesterol was necessary. This could be cholesterol bound to Celite or secreted from cholesterol-loaded macrophages. Second, the presence of active lecithin:cholesterol acyltransferase (LCAT) was required to esterify the

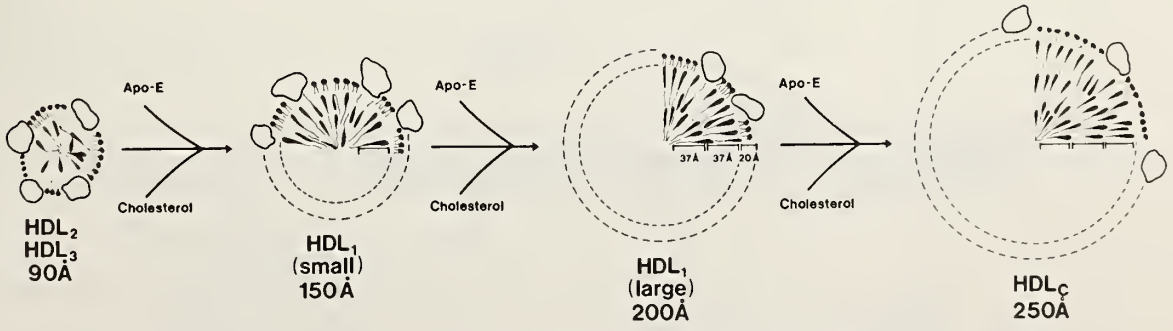

Figure 6. Model of size expansion of canine HDL suggesting how discrete subclasses of HDL could be formed from incubation with an exogenous source of cholesterol. The small (90-Å) HDL contain cholesteryl esters that are randomly distributed in the core of the particle. As the larger HDL are formed by the lecithin:cholesterol acyltranferase-(LCAT-) mediated addition of cholesteryl ester to the core and of apo-E to the surface, the esters appear to arrange themselves in layers. The quantum changes in size are accounted for by the addition of layers of cholesteryl esters to the core.

cholesterol. Third, a source of apo-E was necessary for the expansion of the HDL particles. The apo-E could be derived from lower-density lipoproteins or from macrophages. As the particles became larger, the amount of apo-E increased. The increase in size was due to core expansion of cholesteryl ester by the action of LCAT and to an increase in the thickness of the surface coat as the result of uptake of unesterified cholesterol. Finally, an important point of these studies was the demonstration that these enlarged and apo-E-enriched HDL were capable of effectively binding to apo-B,E(LDL) receptors.

These results support the hypothesis that HDL-with apo-E play an important role in reverse cholesterol transport and in the redistribution of excess cholesterol. As summarized in Figure 7, excess unesterified cholesterol can be acquired from peripheral cells by the small HDL, esterified by LCAT, and packaged into the core. This core expansion is associated with enrichment of the particles with apo-E, giving these HDL-with apo-E the ability to bind to lipoprotein receptors and to deliver cholesterol to cells. If the cells are hepatocytes, this process describes a direct mechanism for reverse cholesterol transport to the liver. An alternative, indirect pathway for the reverse cholesterol transport process would occur in species with high levels of cholesteryl ester exchange. In the indirect pathway, cholesteryl ester in HDL, including HDL-with apo-E, would be transferred to lower-density lipoproteins (VLDL, LDL) by cholesteryl ester transfer proteins, and these lipoproteins would then be

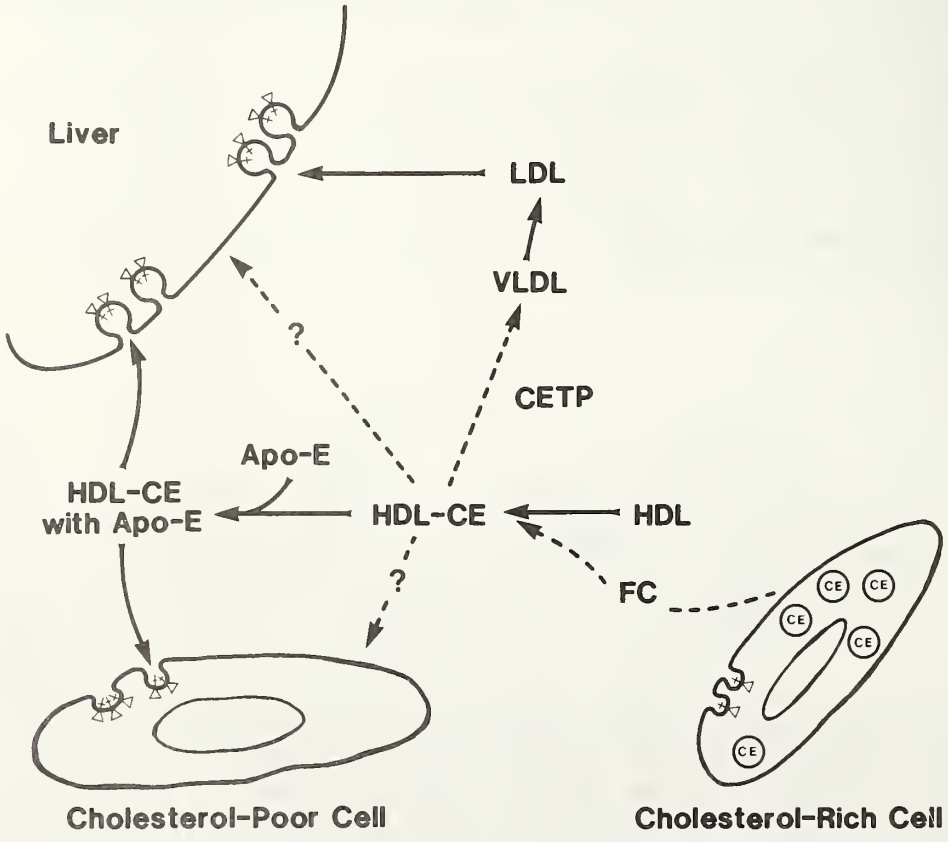

Figure 7. Schematic representation of the role of HDL-with apo-E in the redistribution of cholesterol among hepatic and nonhepatic cells. CETP, cholesteryl ester transport proteins; HDL-CE, cholesteryl ester-enriched HDL; FC, free cholesterol.

removed by hepatic receptors (for review, see reference 30). In species with high cholesteryl ester transfer activity, such as humans, the HDL-with apo-E would play a minor role in reverse cholesterol transport. In humans, the HDL-with apo-E transport approximately 11% of the HDL cholesterol (16).

A second potentially important role for HDL-with E is in cholesterol redistribution. Because the HDL-with apo-E can deliver cholesterol to any cell expressing apo-B,E(LDL) receptors, the delivery of excess peripheral cholesterol transported by HDL-with apo-E is not limited to hepatic cells. Any cholesterol-poor cell expressing receptors is a target for cholesterol delivery by HDL-with apo-E. Delivery of peripherally derived cholesterol to nonhepatic cells would represent redistribution in that the cholesterol would not be directed to the liver for elimination, as it is in reverse cholesterol transport. This redistribution of cholesterol among cells could be a very important *in vivo* process, providing a mechanism for cells with excess cholesterol to transfer it to cholesterol-poor cells among tissues or even within the same tissue.

Summary

Much that has been learned from studies of canine HDL-with apo-E can be applied to human plasma. Although HDL-with apo-E are not as prominent in humans as they are in the dog and other species, they are present in modest concentrations and display a similar size heterogeneity. The lower level of HDL-with apo-E in human plasma is probably related to the higher level of cholesteryl ester transfer activity in humans. Because of the presence of apo-E, HDL-with apo-E can bind to lipoprotein receptors and deliver cholesterol to cells. Therefore, it is likely that HDL-with apo-E play an important role in both cholesterol redistribution and reverse cholesterol transport. In species with high plasma levels of cholesterol transfer activity, their role in reverse cholesterol transport may be both direct and indirect. An additional consideration in humans is the existence of several structural variants of apo-E, a number of which exhibit defective binding to lipoprotein receptors. Finally, the ability of cysteine-containing apo-E to form apo(E—A-II) appears to have a profound effect on the plasma distribution of apo-E and perhaps on plasma levels of LDL.

Acknowledgments

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IN VIVO AND IN VITRO METABOLISM OF INTESTINAL AND HEPATIC REMNANT LIPOPROTEINS: ROLE OF BETA-VERY LOW DENSITY LIPOPROTEINS IN ATHEROSCLEROSIS

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Introduction

Beta-very low density lipoproteins (β-VLDL) were first found by Gofman et al. in the plasma of patients with xanthoma tuberosum (type III hyperlipoproteinemia). These patients had high levels of lipoproteins that exhibited flotation of S_f = 20-400 (VLDL) (1). Fredrickson et al. found that the elevated VLDL showed β mobility on electrophoresis instead of the normal pre-β mobility (2, 3). We now know that the appearance of β-VLDL is a characteristic feature of patients with the genetic abnormality type III hyperlipoproteinemia (4, 5). In addition, Mahley and co-workers found that β-VLDL appear in the plasma of dogs, rats, rabbits, swine, monkeys, as well as humans, fed a high-cholesterol diet (6). The β-VLDL from humans and animals float at the density of plasma (d = 1.006 g/ml) and have β-electrophoretic mobility, whereas normal plasma contains only triglyceride-rich VLDL and no detectable amounts of β-VLDL. In contrast to VLDL, β-VLDL have a core composed largely of cholesteryl ester. The other main difference between VLDL and β-VLDL is a quantitative difference in apolipoprotein content: β-VLDL contain more apolipoprotein (apo-) E and fewer C apolipoproteins than do normal VLDL (4, 5).

All type III hyperlipoproteinemia patients are homozygous for an apo-E allele that codes for a variant apo-E protein characterized by poor binding to lipoprotein receptors (4, 5). The most common receptor-defective form of apo-E is apo-E2, in which cysteine is substituted for arginine at residue 158 (7). However, other neutral amino acids substituted for basic amino acids in the receptor binding domain of apo-E also disrupt binding activity, resulting in the appearance of β-VLDL in the plasma of these subjects (8-10).

The β-VLDL particles are believed to represent cholesterol-rich remnant particles that are created after the triglycerides of VLDL and chylomicrons are hydrolyzed by lipoprotein lipase. Chylomicrons or VLDL bind poorly to hepatic lipoprotein receptors, but after lipolysis the apo-E in these lipoprotein particles becomes more exposed or activated and mediates the binding of the lipoproteins to hepatic lipoprotein receptors. Normally, chylomicron remnants are rapidly cleared by the liver, whereas VLDL remnants are either cleared by the liver or converted to LDL. When the normal hepatic clearance mechanism is either overloaded or functions defectively (in cholesterol-fed animals and in patients with familial type III hyperlipoproteinemia), the remnant particles remain in the plasma as the cholesteryl ester-rich β-VLDL (11-13).

Ross and Zilversmit provided evidence that in cholesterol-fed rabbits β-VLDL are chylomicron remnants that accumulate in the plasma (14), while Swift et al. (15) and Kris-Etherton and Cooper (16) demonstrated that in rats fed cholesterol, β-VLDL can be secreted by the liver. More recently,

Fainaru et al. (17) determined that β-VLDL from cholesterol-fed dogs and from humans with type III hyperlipoproteinemia are heterogeneous and can be subfractionated by agarose gel chromatography into two discrete populations. One population, designated Fraction I β-VLDL, appears to be of intestinal origin, i.e., it is derived from intestinal chylomicrons (see below). The other population, referred to as Fraction II β-VLDL, is probably of hepatic origin. Both subfractions are cholesteryl ester-rich, have β-mobility on Pevikon block electrophoresis, and possess apo-B and apo-E as major apolipoprotein constituents. Fraction I and II β-VLDL differ in size, shape, electrophoretic mobility on paper, chemical composition, and apo-B species. Fraction I β-VLDL are larger in diameter (90 to 300 nm) and have an irregular redundant surface; Fraction II β-VLDL (20 to 70 nm) are spherical, as seen by negative staining electron microscopy. On paper electrophoresis, Fraction I β-VLDL remain at the origin, whereas Fraction II β-VLDL have β-mobility. Compared with Fraction II, Fraction I lipoproteins contain more triglyceride and less protein. Canine and human Fraction II β-VLDL contain predominantly apo-B100 and only small amounts of apo-B48. Canine Fraction I β-VLDL contain equal amounts of apo-B48 and apo-B100, whereas Fraction I β-VLDL from patients with type III hyperlipoproteinemia contain mainly apo-B48.

The origin of each β-VLDL subfraction can be traced by the apo-B composition. In humans, apo-B100 is believed to be synthesized by the liver, and thus the Fraction II β-VLDL are of hepatic origin, whereas apo-B48, and therefore Fraction I β-VLDL, are intestinally derived (17, 18). Very low density lipoproteins from patients with type III hyperlipoproteinemia have been subfractionated by Milne et al. (19), using immunoaffinity chromatography, into apo-B100 β-VLDL and apo-B48 β-VLDL. This demonstrates that apo-B48 and apo-B100 are on different lipoprotein particles.

In Vivo Metabolism of β-VLDL

Our understanding of the catabolism of β-VLDL stems from studies in which ^{125}I-β-VLDL were injected into animals and their metabolic fate was followed in vivo. These studies have shown that β-VLDL particles are rapidly removed from the circulation. In rats, the plasma clearance of canine ^{125}I-β-VLDL was greatly reduced after the lipoproteins had been reductively methylated (20). This selective modification of the lysine residues of apo-B- and apo-E-containing lipoproteins abolishes receptor binding to the apo-B,E(LDL) receptors and to the hepatic apo-E receptor (21, 22). Thus, β-VLDL are cleared from the circulation in part by a receptor-mediated mechanism.

Since β-VLDL are a mixture of at least two main subfractions, meaningful studies on the metabolism of these lipoproteins required the fractionation of canine β-VLDL into Fraction I β-VLDL and Fraction II β-VLDL. When ^{131}I-labeled Fraction I β-VLDL and ^{125}I-labeled Fraction II β-VLDL were injected simultaneously into a normal dog, both fractions were cleared from the plasma very rapidly (<5% of the injected dose remained in the plasma after 2 hr) (Fig. 1A). Almost all of the ^{131}I-labeled Fraction I β-VLDL and ^{125}I-labeled Fraction II β-VLDL could be found in the liver (23).

However, major differences in the clearance mechanism of ^{131}I-labeled Fraction I β-VLDL and ^{125}I-labeled Fraction II β-VLDL could be discerned by the simultaneous injection of both fractions into cholesterol-fed dogs. As shown in Figure 1B, Fraction I β-VLDL were still cleared very rapidly from the circulation. In contrast, the clearance of Fraction II β-VLDL was substantially different from that observed in normal dogs. The plasma clearance of Fraction II β-VLDL was characterized by an initial, rapid die-away of ~40% of the injected dose, followed by a slower disappearance of the radioactivity from the plasma. The divergence of the clearance

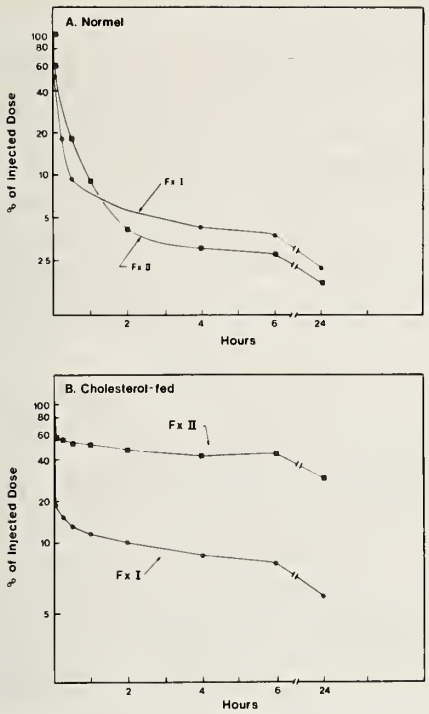

Figure 1. (A) Plasma clearance of canine ^{131}I-Fraction I β-VLDL (●) and ^{125}I-Fraction II β-VLDL (■) in a normal dog. Approximately 0.5 mg of lipoprotein protein of each β-VLDL fraction was injected into the cephalic vein of a normolipidemic foxhound (plasma cholesterol = 150 mg/dl). (B) Plasma clearance of canine ^{131}I-Fraction I β-VLDL (●) and ^{125}I-Fraction II β-VLDL(■) in a cholesterol-fed dog. Approximately 0.5 mg of lipoprotein protein of each β-VLDL fraction was injected into the cephalic vein of a cholesterol-fed foxhound (plasma cholesterol = 790 mg/dl).

times of Fraction I and Fraction II β-VLDL suggested that these two types of particles could be catabolized by two different lipoprotein receptor-mediated pathways. As will be described, the Fraction I β-VLDL are catabolized by chylomicron remnant receptors and the Fraction II β-VLDL are catabolized by hepatic apo-B,E(LDL) receptors.

Kita et al. (24) first demonstrated the possibility of two different hepatic functional pathways. When radioactive rat chylomicron remnants were injected into normal and Watanabe heritable hyperlipidemic (WHHL) rabbits, the lipoprotein particles were rapidly cleared at the same rate. Because WHHL rabbits do not possess functional apo-B,E(LDL) receptors and the clearance of the chylomicron remnants had characteristics of a receptor-mediated process, these studies provided strong evidence that a hepatic lipoprotein receptor distinct from the apo-B,E(LDL) receptor was mediating the clearance of the chylomicron remnants. Arbeeny et al. (25) obtained additional evidence for a functional chylomicron remnant receptor by examining the uptake of rat ^{125}I-labeled chylomicron remnants by the perfused rat liver. In the cholesterol-fed rat, in which the expression of the hepatic apo-B,E(LDL) receptor was down-regulated, chylomicron remnant uptake was only slightly depressed. This experiment suggests the presence of a second receptor (chylomicron remnant receptor) that is not down-regulated by cholesterol feeding. In these studies the uptake of apo-E-deficient remnants amounted to only 25% of the uptake of normal remnants, thereby demonstrating the importance of apo-E in the hepatic uptake of chylomicron remnants; these results are in agreement with previous studies (26). We have isolated a receptor (termed the apo-E receptor) that has the properties of the functional chylomicron remnant receptor (22). Whether or not the apo-E receptor is the chylomicron remnant receptor remains to be determined. Because the clearance of the intestinal chylomicron remnant fraction (Fraction I β-VLDL) was unaltered in the cholesterol-fed dogs, these results most likely reflect the clearance of Fraction I β-VLDL by chylomicron remnant receptors that are not down-regulated by cholesterol feeding.

The clearance of Fraction II β-VLDL was more complex. The difference between the clearance of Fraction II β-VLDL in the normal and cholesterol-

fed dogs was shown to be, for the most part, the result of the down-regulation of the expression of the hepatic apo-B,E(LDL) receptor. Two different approaches demonstrated the importance of hepatic apo-B,E(LDL) receptor for Fraction II β-VLDL catabolism. First, cyclohexanedione modification of the arginine residues of Fraction II β-VLDL, which abolishes the binding of lipoproteins to lipoprotein receptors, was employed (27). Cyclohexanedione modification markedly inhibited the clearance of Fraction II β-VLDL from the circulation of normal dogs, but had little effect on the clearance of Fraction II β-VLDL in cholesterol-fed dogs. The rapid acute phase (initial 10 min) of clearance of the Fraction II β-VLDL in the cholesterol-fed dogs did not appear to depend on the apo-B,E(LDL) receptors (Fig. 2).

The second approach was to use bile acid infusion to modulate hepatic apo-B,E(LDL) receptor expression. Hepatic apo-B,E(LDL) receptors are rapidly down-regulated by taurocholate infusion into dogs (28). In these studies, dogs were infused with two concentrations of taurocholate for 4 or 5 hr to down-regulate the expression of hepatic apo-B,E(LDL) receptors, and then the iodinated Fraction II β-VLDL were injected. The infusion of 4 μmol of taurocholate substantially retarded the clearance of the Fraction II β-VLDL, while the lower concentration (2.5 μmol) resulted in an intermediate clearance of Fraction II β-VLDL compared with that of the control (Fig. 3). These results indicated that Fraction II β-VLDL is catabolized by the hepatic apo-B,E(LDL) receptors.

The initial phase of the uptake of Fraction II β-VLDL does not appear to be receptor-mediated clearance. This initial phase is most likely due to Fraction II β-VLDL binding to endothelial surfaces for lipolytic processing. Fraction II β-VLDL preincubated with post-heparin plasma or intermediate density lipoproteins (IDL, d = 1.006-1.03 g/ml) from a cholesterol-fed dog did not demonstrate the initial rapid die-away when injected into cholesterol-fed dogs (23). In cholesterol-fed dogs the slower catabolism of Fraction II β-VLDL provides a greater opportunity for lipolytic processing by lipoprotein lipase. Indeed, it was observed that when Fraction II β-VLDL were not rapidly cleared from the plasma of cholesterol-fed dogs, the lipoprotein particles were converted to smaller particles that possess the density of IDL and LDL (23). This effect is very similar to that observed for the metabolism of VLDL in WHHL rabbits

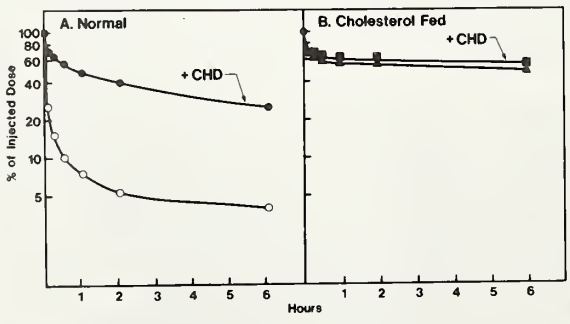

Figure 2. Effect of cyclohexanedione (CHD) modification of arginine residues of canine Fraction II β-VLDL from the plasma of normal (A) and cholesterol-fed dogs (B). The ^{125}I-labeled-CHD-Fraction II β-VLDL (●,■) and the unmodified ^{131}I-labeled Fraction II β-VLDL (○,▲) were injected simultaneously into the same normal (○,●) or cholesterol-fed (■,▲) dog.

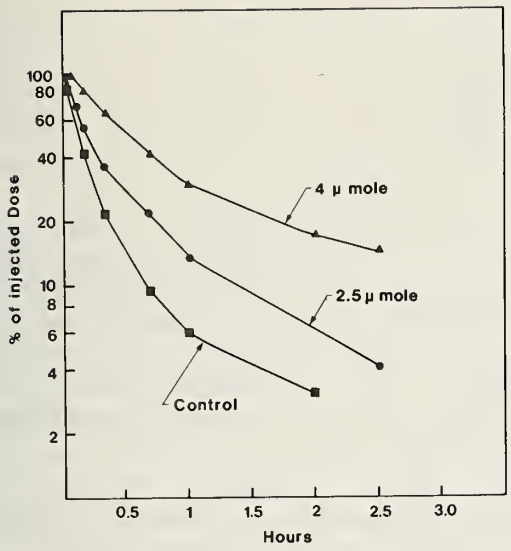

Figure 3. Effect of taurocholate infusion on canine Fraction II β-VLDL clearance from the plasma of a normolipidemic dog. The plasma clearance of the ^{125}I-Fraction II β-VLDL in the dog receiving 2.5 (●) or 4.0 (▲) μmol of taurocholic acid/kg of body weight/min is compared to the clearance of ^{125}I-Fraction II β-VLDL (■) in a saline-infused normal dog.

(29). After the injection of ^{125}I-VLDL into normal and WHHL rabbits, the uptake of ^{125}I-apo-B by the liver and the conversion of VLDL to IDL and LDL were measured. In normal rabbits, the VLDL were more rapidly cleared from the plasma than were the VLDL injected into WHHL rabbits. In the WHHL rabbits, which possess low levels of functional apo-B,E(LDL) receptors, there was a much greater conversion of the injected VLDL to IDL and LDL than in the control animals. These studies led Kita et al. to postulate that a decrease in the number of hepatic LDL receptors leads to an enhanced conversion of VLDL to IDL and LDL and thus an overproduction of LDL. At the same time the catabolism of LDL by the liver would be retarded because of the deficiency of functional hepatic LDL receptors.

The studies using Fraction II β-VLDL indicate that the hepatic apo-B,E(LDL) receptors are responsible for clearance of the Fraction II β-VLDL. These studies also support the concept that the number of LDL receptors expressed by the liver plays a major role in determining the fraction of VLDL remnants (Fraction II β-VLDL) converted to IDL and LDL vs. the fraction of these lipoprotein particles cleared from the plasma by the liver without conversion to other lipoproteins. In cholesterol-fed dogs, the down-regulation of the LDL receptor by cholesterol feeding (5) prevents the normal clearance of Fraction II β-VLDL and results in the conversion of these particles to cholesterol-rich IDL and LDL. Thus, because of cholesterol feeding, there is an acquired deficiency of LDL receptors that causes the same alteration of cholesterol metabolism as a genetic deficiency of apo-B,E(LDL) receptors (Fig. 4).

In Vitro Metabolism of β-VLDL

When mouse peritoneal macrophages are incubated with β-VLDL, the lipoproteins are taken up by a receptor-mediated process and degraded in the lysosomes. The liberated cholesterol enters the cytoplasm, where is it re-esterified by the microsomal enzyme acyl CoA:cholesterol acyltransferase (ACAT). When [^{14}C]oleate is added to the culture medium, the cells incorporate the oleate into cholesteryl [^{14}C]oleate. The addition of β-VLDL to the medium leads to a 100- to 200-fold increase in the rate of conversion of [^{14}C]oleate into cholesteryl [^{14}C]oleate. In addition, macrophages incubated with β-VLDL show a 20- to 160-fold increase in the mass of cellular cholesteryl ester and histologically resemble aortic foam cells (30, 31). Of all the naturally occurring plasma lipoproteins, β-VLDL are the only lipoproteins that induce a massive increase in cholesterol

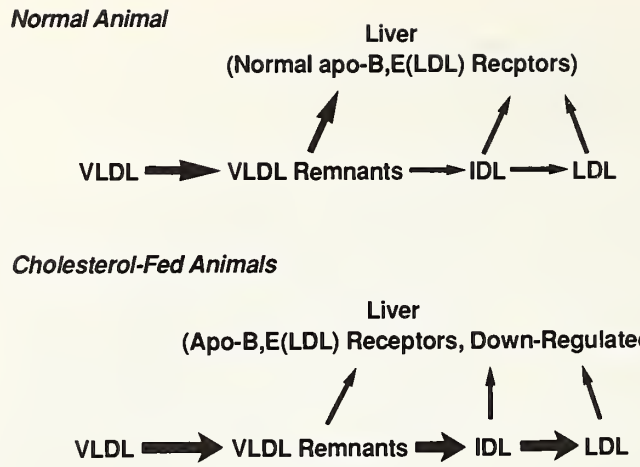

Figure 4. Schematic diagram illustrating the effect of the down-regulation of hepatic apo-B,E(LDL) receptors on VLDL remnant catabolism.

esterification and cytoplasmic cholesteryl esters when incubated with mouse peritoneal macrophages. Since many, if not most, aortic foam cells are derived from monocyte macrophages, these results demonstrated a possible mechanism for macrophage foam cell formation (32).

Ho, Brown, and Goldstein have shown that HDL can promote the efflux of cholesterol from macrophages previously loaded with cholesterol (33). Furthermore, we have shown that β-VLDL and low concentrations of HDL act synergistically to modulate the cholesteryl ester concentration in macrophages. When mouse peritoneal macrophages were incubated with β-VLDL (100 μg of cholesterol/ml), 65 μg of cholesteryl ester sterol/mg of macrophage protein was observed. The addition of 10 μg of HDL cholesterol to the medium along with the β-VLDL decreased the mass of cholesteryl ester to 22 mg of sterol/mg of cellular protein. Thus, it is reasonable to speculate that an important determinant of whether cholesteryl esters accumulate in the macrophage model system, and possibly the arterial wall, is the ratio of lipoproteins capable of delivering cholesterol to the cells (β-VLDL, modified LDL) to those capable of removing cholesterol from the cells (HDL) (34).

Receptor for β-VLDL. Initial studies indicated that the uptake of β-VLDL by mouse peritoneal cells was mediated by a lipoprotein receptor (27, 28). The receptor for β-VLDL appeared to be distinct from the classic apo-B,E(LDL) receptor that has been so elegantly described on human fibroblasts and other tissues. This conclusion was based on the observations that mouse macrophages bind and internalize minimal amounts of normal human LDL and that incubation of mouse macrophages with LDL does not lead to appreciable cholesteryl [^{14}C]oleate formation or to the accumulation of cholesteryl esters within the cells. Second, human LDL were not effective in competing with canine ^{125}I-β-VLDL for receptor-mediated degradation. Finally, the receptor on mouse peritoneal macrophages that mediated the uptake of β-VLDL was relatively resistant to down-regulation by prior incubation of the cells with human LDL. Because the properties of the macrophage receptor differed so dramatically from those of the classic apo-B,E(LDL) receptor, a separate receptor was postulated, and this receptor was designated the β-VLDL receptor (13).

In addition to the initial studies that suggested the presence of a distinct β-VLDL receptor, three other laboratories presented evidence supporting the concept of a distinct β-VLDL receptor in macrophages. In studies using hypertriglyceridemic very low density lipoproteins (HTG-VLDL), Gianturco and Bradley's results indicated the presence of the β-VLDL receptor on mouse peritoneal macrophages (35). They showed that HTG-VLDL would compete with rabbit ^{125}I-β-VLDL for uptake by mouse

macrophages, suggesting the HTG-VLDL particles were binding to the β-VLDL receptor. Uptake of HTG-VLDL by the cells delivered triglycerides, which were hydrolyzed by the lysosomes. The free fatty acids were re-esterified, producing a massive triglyceride engorgement and foam cell morphology. These authors concluded that HTG-VLDL and β-VLDL bind to the same receptor and proceed through the same intracellular pathway (35).

The strongest evidence for a distinct β-VLDL receptor was provided from experiments using monocyte-derived macrophages from a patient with the homozygous form of familial hypercholesterolemia (FH). The human fibroblasts from this patient had defective apo-B,E(LDL) receptors, which bound only ~5% of the LDL bound by apo-B,E(LDL) receptors on normal fibroblasts. The monocyte-macrophages from this individual degraded rabbit ^{125}I-β-VLDL normally, which indicated that an additional, genetically separate receptor must be responsible for the cellular uptake and degradation of the ^{125}I-β-VLDL (36). In a similar experiment, Wang-Iverson et al. (37) demonstrated that normal VLDL can also be metabolized by monocyte-macrophages from subjects with FH.

Receptor for β-VLDL on Mouse Macrophages. With the long-range goal of isolating the β-VLDL receptor, we undertook a thorough examination of the properties of the β-VLDL receptor. The first series of studies was to clarify the apolipoprotein specificity of the β-VLDL receptor. As noted, β-VLDL possess apo-B and apo-E as the major apolipoproteins, suggesting that one of these must mediate binding to the receptor; in fact, the apolipoprotein responsible for the uptake of β-VLDL has been determined to be apo-E (38). It was shown that canine apo-E HDL$_c$ (cholesterol-rich HDL containing only apo-E) were able to inhibit totally the cellular uptake and degradation of ^{125}I-β-VLDL. Moreover, when the lysine residues of the apolipoproteins on β-VLDL were reductively methylated, which abolishes receptor binding, the β-VLDL were not taken up by the macrophages. After native apo-E was reconstituted in the β-VLDL in place of reductively methylated apo-E, the β-VLDL were avidly taken up by the cells. In the reverse experiment, in which reductively methylated apo-E was incorporated into the unmodified β-VLDL particle, the lipoproteins were not taken up or degraded by the macrophages (38). Independently, Bates et al. (39), using mouse peritoneal macrophages and J774 cells, and Van Lenten et al. (40), using rabbit β-VLDL and human monocyte-macrophages, identified apo-E as the receptor binding determinant of β-VLDL.

To characterize the lipoprotein receptors on mouse macrophages, antibody and binding studies were combined. Using a specific polyclonal antibody raised against the bovine apo-B,E(LDL) receptor, we determined that a single band in Triton X-100 extracts of mouse macrophages could be detected on immunoblots (41). The immunoreactive band has the same apparent molecular weight as the apo-B,E(LDL) receptor on mouse fibroblasts (Fig. 5), which is ~5000 less than that of human and bovine apo-B,E(LDL) receptor. Ligand blot analysis using ^{125}I-β-VLDL also detected a single band on SDS-polyacrylamide gel electrophoresis, i.e., the apo-B,E(LDL) receptor (Fig. 5). Very recently, Ellsworth et al. (42) have obtained identical results using specific antibodies against the rat liver apo-B,E(LDL) receptor. They found that the mouse peritoneal macrophages, the mouse macrophage cell line J774, and the mouse fibroblast cell line 3T3 possess an apo-B,E(LDL) receptor with a molecular weight ~5000 less than that of rat liver apo-B,E(LDL) receptor (42).

Three major lines of evidence indicate that the apo-B,E(LDL) receptor on mouse peritoneal macrophages is responsible for the high-affinity uptake of β-VLDL by these cells. First, binding studies at 4°C demonstrated specific and saturable binding of human ^{125}I-LDL, canine ^{125}I-β-VLDL, and canine ^{125}I-apo-E HDL$_c$ to the apo-B,E(LDL) receptor on mouse peritoneal

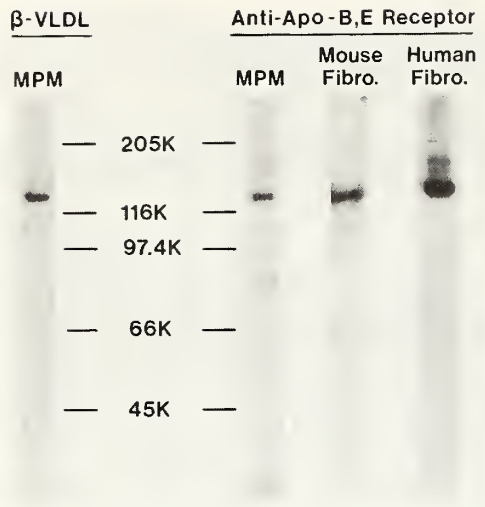

Figure 5. Ligand blot (left) and immunoblot (right) of apo-B,E(LDL) receptors on human fibroblasts and mouse peritoneal macrophages and fibroblasts. The ligand blot was performed using 1 μg of ^{125}I-β-VLDL protein/ml. The immunoblot was reacted with rabbit polyclonal anti-apo-B,E(LDL) receptor, followed by ^{125}I-labeled donkey anti-rabbit immunoglobulin G.

macrophages. The human ^{125}I-LDL bound to mouse macrophages with an 18-fold lower affinity than to human fibroblasts. However, the apo-E-containing lipoproteins ^{125}I-β-VLDL and ^{125}I-apo-E HDL$_c$ bound to mouse peritoneal macrophages and human fibroblasts with the same very high affinity (41). Competitive binding studies at 4 C indicated that human LDL and canine β-VLDL were both binding to the apo-B,E(LDL) receptor on mouse macrophages. Human, canine, and mouse LDL, as well as canine β-VLDL, totally inhibited the binding of canine ^{125}I-β-VLDL to cultured mouse macrophages and human fibroblasts (Fig. 6). Because LDL bound with a low affinity to the apo-B,E(LDL) receptor on mouse macrophage monolayers, about 100-fold more LDL than β-VLDL were needed to inhibit ^{125}I-β-VLDL binding by 50% (Fig. 6). Competitive binding studies at 37 C illustrated that human LDL were able to inhibit almost all of the cellular uptake of rabbit ^{125}I-β-VLDL and rat ^{125}I-labeled chylomicron remnants by the LDL receptors on mouse J774 macrophages (42). An 800- to 1000-fold excess of human LDL reduced the uptake of rabbit ^{125}I-β-VLDL and rat ^{125}I-labeled chylomicron remnants by 80 to 90% (42).

The second line of evidence that indicated the uptake of ^{125}I-β-VLDL by mouse peritoneal macrophages was mediated by the apo-B,E(LDL) receptor was the inhibition of β-VLDL binding and uptake by antibodies to the apo-B,E(LDL) receptor. Preincubation of the mouse macrophages with an anti-bovine-apo-B,E(LDL) receptor antibody produced a dose-dependent inhibition of both β-VLDL- and LDL-induced cholesterol esterification (41). In addition, anti-rat-apo-B,E(LDL) receptor inhibited the cellular uptake of ^{125}I-β-VLDL and ^{125}I-labeled chylomicron remnants by mouse peritoneal

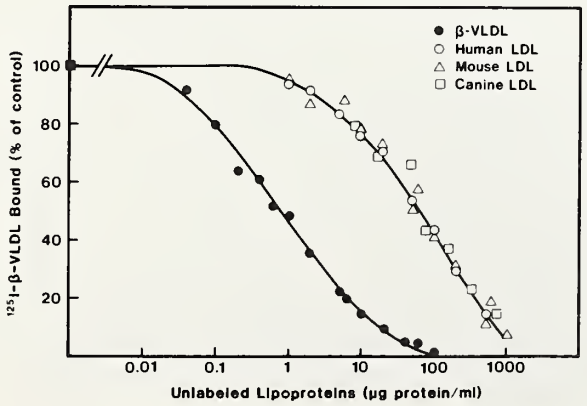

Figure 6. Ability of canine β-VLDL and human, mouse, and canine LDL to compete with ^{125}I-β-VLDL for binding to mouse peritoneal macrophages. Binding experiments were performed with 1 μg of ^{125}I-β-VLDL protein/ml and variable amounts of unlabeled lipoproteins.

macrophages and J774 macrophages (42). The mouse macrophage cell line $P388D_1$ has been reported to have very few apo-B,E(LDL) receptors (without induction of the receptors by incubation in lipoprotein-deficient medium); however, the anti-bovine-apo-B,E(LDL) receptor antibody also totally inhibited the binding of canine ^{125}I-β-VLDL to these cells (Fig. 7).

The third line of evidence that pointed to the apo-B,E(LDL) receptor as the receptor responsible for the uptake of β-VLDL was the lipoprotein-induced down-regulation of β-VLDL binding. Compared with the apo-B,E(LDL) receptor on human fibroblasts, the apo-B,E(LDL) receptors on cultured mouse macrophages were resistant to down-regulation by incubation of the cells with LDL or β-VLDL. However, the mouse peritoneal macrophages in which the apo-B,E(LDL) receptors had been down-regulated displayed decreased binding of ^{125}I-β-VLDL. There was an excellent correlation between the relative cellular content of apo-B,E(LDL) receptors, as determined by immunoblots, and the amount of ^{125}I-β-VLDL bound to the cells (Fig. 8). Ellsworth et al. down-regulated the apo-B,E(LDL) receptor by preincubation of J774 macrophages with 25-hydroxycholesterol. They found the amount of cellular uptake of rabbit ^{125}I-β-VLDL was decreased in parallel with the relative cellular content of apo-B,E(LDL) receptors as measured by immunoblots or by ligand blots using ^{125}I-β-VLDL or ^{125}I-labeled chylomicron remnants (42).

These results from two independent studies indicate that the β-VLDL receptor, as originally described (27, 28), is an unusual LDL receptor. This receptor is the predominant (if not the only) receptor responsible for the high-affinity uptake of canine or rabbit β-VLDL and rat chylomicron remnants by mouse peritoneal macrophages and mouse macrophage cell lines. These results do not exclude the possibility that, under different conditions, a lipoprotein receptor distinct from the apo-B,E(LDL) receptor might be expressed that recognizes β-VLDL. However, our results (41) and those of Ellsworth et al. (42) would indicate that the vast majority of apo-E-containing lipoproteins are taken up by the apo-B,E(LDL) receptor on mouse macrophages.

These studies clarify the misconceptions in the literature about the presence or absence of the apo-B,E(LDL) receptor on mouse macrophages (13). Because of the low affinity of LDL for the apo-B,E(LDL) receptor on mouse macrophages, LDL do not appear to compete effectively against ^{125}I-β-VLDL or other apo-E-containing lipoproteins for receptor binding in competitive binding studies. For example, a 25-fold excess of human LDL did not inhibit the cellular uptake of ^{125}I-labeled hypertriglyceridemic VLDL ($HTG\text{-}VLDL_1$) by mouse macrophages (43). Since $HTG\text{-}VLDL_1$ bind to the apo-B,E(LDL) receptor via apo-E, and because of the great differences in the binding affinity of apo-E-containing lipoproteins and LDL for the

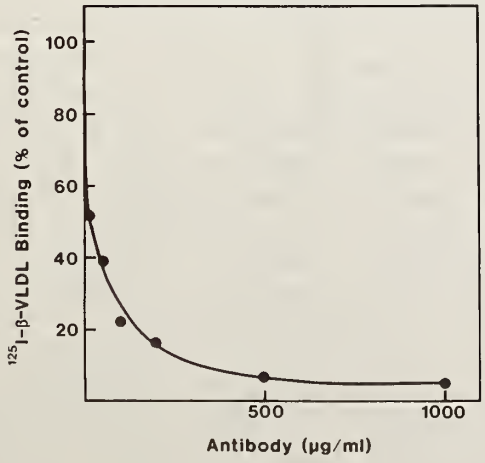

Figure 7. Ability of anti-apo-B,E(LDL) receptor to inhibit the binding of canine ^{125}I-β-VLDL to murine $P388D_1$ cells. The cells were preincubated with anti-apo-B,E(LDL) receptor for 1 hr at 37°C, and binding assays were performed at 4°C using 1 μg of ^{125}I-β-VLDL protein/ml.

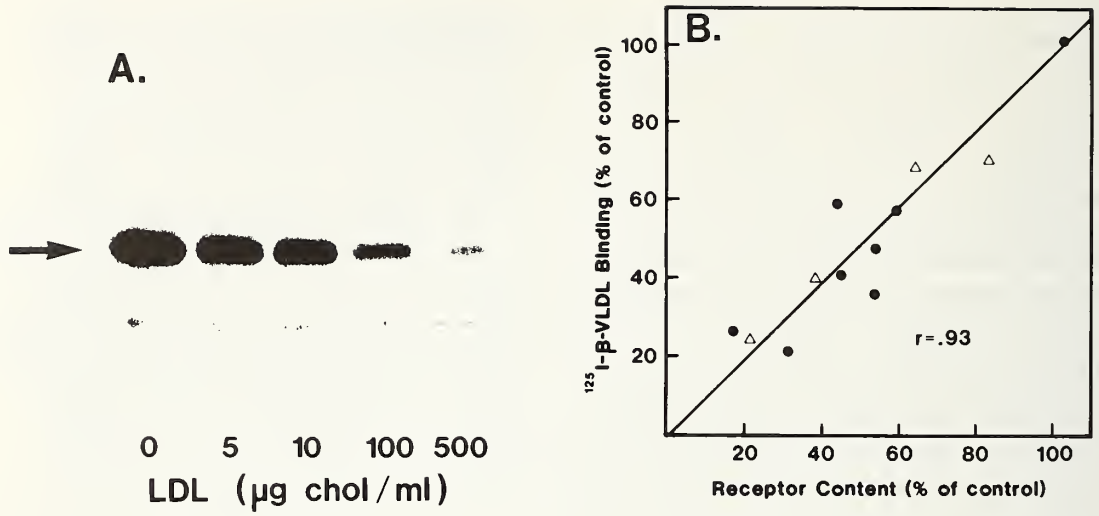

Figure 8. (A) Immunoblots using anti-apo-B,E(LDL) receptor demonstrating the LDL-induced down-regulation of apo-B,E(LDL) receptors (arrow) in mouse peritoneal macrophage extracts. (B) Correlation between canine ^{125}I-β-VLDL binding and the quantity of apo-B,E(LDL) receptors in mouse peritoneal macrophages. The data represented by the circles and triangles are from five experiments. The triangles represent data from the experiment shown in (A). The relative receptor quantity was calculated from densitometric scans of the resulting autoradiographs. Parallel binding experiments were performed using 1 µg of ^{125}I-β-VLDL protein/ml. r, correlation coefficient.

apo-B,E(LDL) receptors on mouse macrophages (a 100-fold difference), a 25-fold excess of LDL is not sufficient to displace the HTG-VLDL$_1$ from the mouse macrophage apo-B,E(LDL) receptors. Another unusual property is that, for reasons not fully understood, the uptake of LDL by mouse peritoneal macrophages does not result in cholesteryl ester formation and accumulation by these cells. However, from these studies it is clear that the mouse macrophages contain an unusual apo-B,E(LDL) receptor that differs from the classic LDL receptor in its lower affinity for LDL and its resistance to down-regulation by extracellular cholesterol.

Other Studies on the Receptor for β-VLDL on Mouse Macrophages. Gianturco et al. examined the in vitro uptake of HTG-VLDL and have concluded that these lipoproteins and β-VLDL do not bind to the apo-B,E(LDL) receptor on mouse peritoneal macrophages, but to a distinct β-VLDL receptor (44). They obtained the following results: When β-VLDL or HTG-VLDL are treated with thrombin, part or most of the apo-E is cleaved into two fragments. Thrombin also processes part of the apo-B100 into fragments similar to apo-B26, apo-B48, and apo-B74. The cleavage of apo-E destroys the ability of either lipoprotein particle to bind to the apo-B,E(LDL) receptor on normal human fibroblasts. However, thrombin-treated HTG-VLDL are taken up at the same rate or at an enhanced rate by mouse macrophages, suggesting that uptake is not mediated by the apo-B,E(LDL) receptor (44). Moreover, the total removal of apo-E with trypsin did not prevent uptake of HTG-VLDL by macrophages. Incorporation of apo-E into the apo-E-depleted HTG-VLDL restored the ability of HTG-VLDL particles to bind to the apo-B,E(LDL) receptor on human fibroblasts. However, the uptake of the HTG-VLDL by macrophages was reduced to original

levels. Gianturco and co-authors interpreted these results as follows: 1) β-VLDL and HTG-VLDL are taken up by a receptor other than the apo-B,E(LDL) receptor, and this receptor is the β-VLDL receptor; and 2) intact apo-E is not required for the uptake of β-VLDL or HTG-VLDL. Therefore, since the major apolipoproteins of HTG-VLDL and β-VLDL are apo-B and apo-E (45), apo-B or apo-B partially hydrolyzed by trypsin or thrombin appears to be required for binding.

Another interpretation of the data is based on the premise that native and thrombin- or trypsin-treated β-VLDL and HTG-VLDL are not taken up by the same receptor: native β-VLDL and HTG-VLDL are taken up by the apo-B,E(LDL) receptor, whereas proteolyzed or processed β-VLDL and HTG-VLDL are taken by another receptor or process. As noted, native β-VLDL and apo-E HDL_c are taken up by an unusual apo-B,E(LDL) receptor on mouse peritoneal macrophages, not by a distinct β-VLDL receptor. The apo-B,E(LDL) receptor binds apo-E-containing lipoproteins with high affinity, and the binding of β-VLDL to this receptor is mediated by apo-E. Presumably, native HTG-VLDL also bind to this unusual apo-B,E(LDL) receptor. Modification of the β-VLDL or HTG-VLDL lipoprotein particles with trypsin or thrombin destroys or greatly reduces their ability to bind to apo-B,E(LDL) receptors on cultured fibroblasts or mouse macrophages.

Our interpretation of the results of Gianturco et al. is that the proteolyzed β-VLDL and HTG-VLDL are metabolized by a process independent of the apo-B,E(LDL) receptor and thus distinct from the receptor-mediated pathway that takes up and degrades native β-VLDL and HTG-VLDL. Macrophages possess a receptor for thrombin (46), and Bradley et al. (47) have shown that highly purified HTG-VLDL contain thrombin or a protease with thrombin-like specificity, which could be recognized by the thrombin receptor. Another possibility is that once these large lipid-rich lipoprotein particles are modified by thrombin or trypsin treatment, the conformation of the apolipoproteins is altered or a new domain is exposed. The altered tertiary structure or exposed domain could be recognized by a distinct receptor on macrophages. Haberland and Fogelman (48) have demonstrated a precedent for this event with their work on maleyl albumin. They found that a conformational change in albumin was necessary for recognition of this protein by the scavenger receptor on human monocyte-macrophages.

Receptor for β-VLDL on Human Monocyte-Macrophages. In 1983, Van Lenten et al. (36) described the uptake and degradation of rabbit β-VLDL by human monocyte-macrophages. These features were noted: 1) The process was dependent on Ca^{2+}, 2) the uptake of LDL and β-VLDL was highest soon after the monocytes were cultured and tended to decline with time in culture, 3) the uptake of both lipoproteins was mediated by a specific high-affinity process, 4) the process was up-regulated by lipoprotein-deficient serum in the medium and down-regulated by preincubation of the cells with sterol in the medium, and 5) the receptor activity recognizes lipoproteins (at least in part) by the apo-E content (40). There was, however, one key difference between the degradation of LDL and β-VLDL by the human monocytes. In monocytes from a homozygous familial hypercholesterolemic patient, the amount of ^{125}I-labeled rabbit β-VLDL that was degraded was only slightly reduced compared with that of normal human monocytes (36). In contrast, the amount of ^{125}I-labeled human LDL degraded was greatly reduced in these cells. These results were interpreted as confirmation of the suggestion by Goldstein et al. (30) that the receptor recognizing β-VLDL was a genetically distinct receptor.

In addition to the monocyte-macrophages from patients with familial hypercholesterolemia, Van Lenten et al. (40) also investigated the β-VLDL receptor on WHHL rabbit alveolar macrophages, which have very low levels of functional apo-B,E(LDL) receptors. Although human ^{125}I-LDL were not taken

up, large amounts of rabbit ^{125}I-β-VLDL were degraded. In a separate study, Baker et al. (49) examined the lipoprotein receptors on aortic endothelial cells. Normal endothelial cells degraded human ^{125}I-LDL, whereas the WHHL aortic endothelial cells did not. However, large amounts of ^{125}I-labeled rabbit β-VLDL were degraded by the normal and WHHL cells (49). These studies were interpreted as providing additional evidence for a distinct β-VLDL receptor.

Detailed studies of the receptors on human monocyte-macrophages were undertaken to attempt to clarify potential differences between mouse and human cells with respect to the presence of β-VLDL receptors. The receptor binding of canine ^{125}I-β-VLDL to human monocyte-macrophages and cultured human fibroblasts have similar, if not identical, properties. The binding affinities of β-VLDL to both cell types were almost identical (50). In addition, the binding of canine ^{125}I-β-VLDL to the human monocyte-macrophages could be totally inhibited by unlabeled human LDL (50). To establish that the apo-B,E(LDL) receptor was responsible for β-VLDL binding in human monocyte-macrophages, a specific polyclonal antibody made against the bovine adrenal apo-B,E(LDL) receptor was used. As shown in Figure 9, the anti-apo-B,E(LDL) receptor antibody inhibited the degradation of both human ^{125}I-LDL and canine ^{125}I-β-VLDL in the human monocyte-macrophages and the human fibroblasts. Immunoblots of the protein extract from human monocytes and human fibroblasts showed a single band with an apparent molecular weight identical to that of the apo-B,E(LDL) receptor as identified using the same antibody (50).

As previously discussed, the apo-B,E(LDL) receptors on mouse peritoneal macrophages were much less efficiently down-regulated by incubation of the cells with apo-B- and apo-E-containing lipoproteins or oxygenated sterols than were the apo-B,E(LDL) receptors on human fibroblasts. To determine whether the apo-B,E(LDL) receptors on human monocyte-macrophages were also poorly down-regulated, human monocyte-macrophages and human fibroblasts were incubated with increasing concentrations of β-VLDL for 48 hr and binding of ^{125}I-β-VLDL to the cells was measured. Both human monocyte-macrophages and human fibroblasts showed equivalent reduction of ^{125}I-β-VLDL binding (Fig. 10). Taken together, these results strongly indicate that human monocyte-macrophages do not possess a distinct β-VLDL receptor. Instead, the receptor on these cells that recognizes human and canine β-VLDL is the apo-B,E(LDL) receptor. Unlike the apo-B,E(LDL) receptor on mouse macrophages, the characteristics of the apo-B,E(LDL) receptor on human monocyte-macrophages appear to be similar, if not identical, to those of the apo-B,E(LDL) receptor on normal human fibroblasts.

To confirm and extend these results, the uptake of β-VLDL by monocyte-macrophages with the homozygous form of FH was reinvestigated. The cells

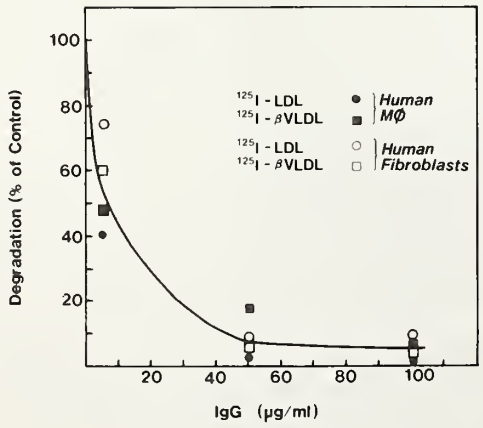

Figure 9. Ability of anti-apo-B,E(LDL) receptor antibody to inhibit the degradation of human ^{125}I-LDL and canine ^{125}I-β-VLDL by human monocyte macrophages (MØ) and human fibroblasts. Cells were preincubated with the antibody for 1 hr at 37°C prior to the addition of ^{125}I-labeled lipoproteins.

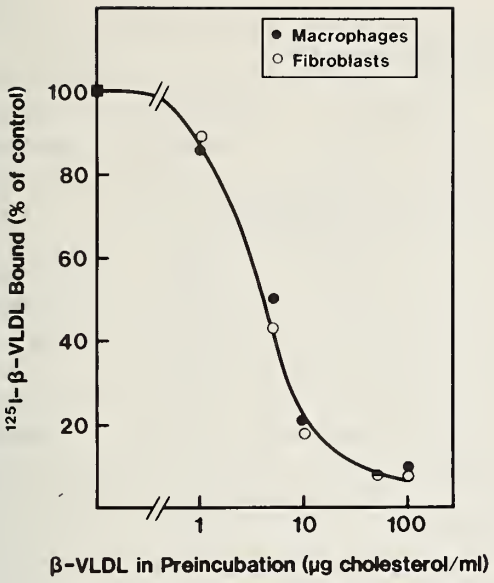

Figure 10. Decreased binding of ^{125}I-LDL and ^{125}I-β-VLDL to human monocyte-macrophages or fibroblasts after preincubation of the cells with various concentrations of β-VLDL. Before the 4°C binding experiment, the cells were washed and further incubated at 37°C for 1 hr to allow internalization of remaining surface-bound β-VLDL.

from these individuals had either low levels of apo-B,E(LDL) receptors or none at all. As shown in Figure 11, canine β-VLDL were not degraded and did not induce cholesteryl ester formation when incubated with monocyte-macrophages from a patient with FH. The use of reductively methylated β-VLDL demonstrated that the uptake and degradation of native β-VLDL by normal monocyte-macrophages were receptor-mediated. The uptake and degradation of canine, rabbit, or human type III FH ^{125}I-β-VLDL or of normal human ^{125}I-LDL by monocyte-macrophages from seven FH homozygous patients were all greatly reduced or absent (Table 1A). Likewise, when the abilities of these lipoproteins to induce cholesterol esterification in normal and FH monocyte-macrophages were compared, very little cholesteryl [^{14}C]oleate was formed in the FH cells (Table 1B) (50). Incubation of acetoacetylated LDL (AcAc LDL) produced roughly equivalent amounts of proteolytic degradation and cholesteryl ester formation in normal and FH cells. The AcAc LDL are taken up by the scavenger (acetyl LDL) receptor, not the apo-B,E(LDL) receptor.

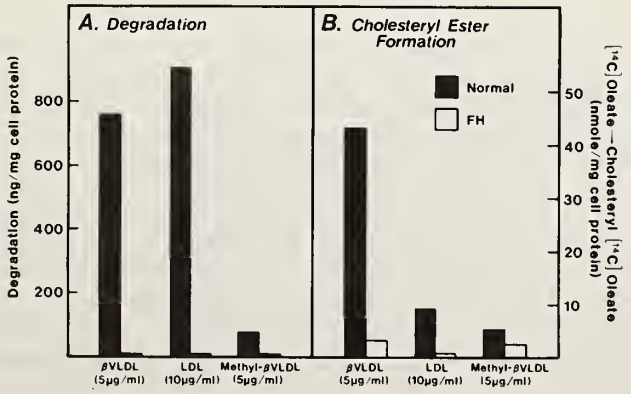

Figure 11. Comparison of the degradation (A) and cholesteryl ester formation (B) in normal monocyte-macrophages and in those from patients with familial hypercholesterolemia (FH) after incubation of the cells with canine β-VLDL, human LDL, or canine methyl-β-VLDL.

Table IA. Degradation of ^{125}I-lipoproteins by human monocyte-macrophages from patients with familial hypercholesterolemia.

Patient	LDL	Canine β-VLDL	Rabbit β-VLDL	Human β-VLDL	AcAc LDL[1]
			(% of normal control)		
F1	1.3	1.6	ND[2]	ND	ND
F6	2.8; 4.9	15.6; 12.5	ND	ND	ND
F8	9.2	2.6	14	ND	ND
2	0.4	0.2	1; 0	0.1; 0	144; 133
3	0.5	ND	3.1	9.1	481
7	3.3	2.0	8.6	8.6	198
9	0.4	ND	1.5	0	142

[1] Acetoacetylated LDL.
[2] ND, not determined.

Table IB. Lipoprotein-induced cholesterol esterification by human monocyte-macrophages of familial hypercholesterolemia patients.

Patient	LDL	Canine β-VLDL	Rabbit β-VLDL	Human β-VLDL	AcAc LDL[1]
			(% of normal control)		
F1	7.6	7.6	ND[2]	ND	ND
F6	0	1.8; 0.1	ND	ND	ND
F8	2.0	6.0	7.5	ND	ND
2	2.3	3.9	4.9; 3.6	4.9; 4.0	119
3	0	ND	0	0	ND
7	0	0	1.2	0	113
9	0	ND	1.6	0	128

[1] Acetoacetylated LDL.
[2] ND, not determined.

The reason for the differences between the results reported here and those from previous studies is not clear, but there are several possibilities. The FH homozygous patient used in the Van Lenten et al. studies had about 5% of normal apo-B,E(LDL) receptor activity, and therefore the apo-B,E(LDL) receptor was defective but not absent. Hobbs et al. (51) have recently described the structural and binding characteristics of the apo-B,E(LDL) receptor from a FH homozygous patient that bound almost no human LDL but interacted with rabbit β-VLDL with the typical high affinity observed with the normal apo-B,E(LDL) receptor. It is possible that Van Lenten et al. (36) and Wang-Iverson et al. (37) used FH monocytes that possessed defective LDL receptors that bound LDL poorly yet still retained their ability to bind and take up β-VLDL. Differences in lipoproteins or experimental conditions may also account for the degradation of β-VLDL in FH monocytes in the previous studies. However, the data presented in our recent studies demonstrate that LDL and β-VLDL degradation were reduced in parallel for seven individuals with FH and verify that the apo-B,E(LDL) receptor mediates β-VLDL metabolism in human monocyte-macrophages (50).

Summary

Beta-very low density lipoproteins (β-VLDL) are cholesterol- and apo-E-rich lipoproteins that float at d < 1.006 g/ml upon centrifugation and have β-electrophoretic mobility. The β-VLDL are either chylomicron remnants (Fraction I) or VLDL remnants (Fraction II) that accumulate in the plasma of subjects with type III hyperlipoproteinemia or of cholesterol-fed animals. Type III subjects have an abnormal apo-E that binds poorly to hepatic lipoprotein receptors; as a consequence of this defective interaction, the remnant particles accumulate in the plasma as β-VLDL. In cholesterol-fed animals, an overproduction of chylomicron and VLDL remnants and a down-regulation of hepatic LDL receptors results in the build-up of β-VLDL. In vivo studies in dogs demonstrate that Fraction I β-VLDL are cleared from the plasma by the liver, apparently by the chylomicron remnant receptor. The clearance of Fraction II β-VLDL is mediated by the hepatic apo-B,E(LDL) receptor; when these receptors are down-regulated in cholesterol-fed animals, much of the Fraction II β-VLDL are converted to IDL and LDL. The β-VLDL are associated with accelerated atherosclerosis in cholesterol-fed dogs and in type III hyperlipoproteinemic patients and are the only naturally occurring lipoproteins that cause massive accumulation of cholesteryl esters when incubated in vitro with macrophages.

Whereas a distinct β-VLDL receptor was initially proposed as mediating the uptake of β-VLDL, it now appears that an unusual LDL receptor on mouse peritoneal macrophages is responsible for the receptor-mediated uptake of β-VLDL. In addition, the apo-B,E(LDL) receptor on human monocyte-macrophages mediates the uptake of human, rabbit, and canine β-VLDL. In contrast to the mouse macrophages, the apo-B,E(LDL) receptor on human macrophages appears to be identical to the apo-B,E(LDL) receptor on human fibroblasts. Monocytes from familial hypercholesterolemic homozygotes that have no functional LDL receptors failed to take up or degrade β-VLDL. The mechanism responsible for the accumulation of cholesteryl esters in macrophages in response to β-VLDL remains to be defined.

Acknowledgments

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HETEROGENEITY OF HUMAN PLASMA Lp(a)

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Introduction

Lipoprotein(a), Lp(a), is the terminology introduced in 1963 by Berg (1) to designate an antigen which he found to be distinct from the Ag system and to be present in 10-30% of human subjects screened by double immunodiffusion using antisera raised in the rabbit and absorbed with $Lp(a)^-$ serum. Subsequent studies have shown that Lp(a) has pre-beta mobility on agarose gel electrophoresis, a hydrated density ranging between 1.05 and 1.12 g/ml and sediments at a medium density of 1.063 g/ml, "sinking pre-beta" (2-4). Based on his original screening technique, Berg classified the subjects studied as $Lp(a)^+$ and $Lp(a)^-$. However, it is difficult to determine whether the reported $Lp(a)^-$ subjects were truly negative. The assessment of these past studies is hampered by differences in antigenic preparations, antibodies and assay techniques used and also by the limited knowledge then available on the properties of the apo(a) antigen. Later investigators using highly sensitive immunoassay techniques concluded that small amounts of Lp(a) are present in the plasma of all human subjects (5). However, none of these assays address the fact that Lp(a) is heterogenous, an observation originally reported by Harvie and Schultz (6). Recent technical developments have made it possible to gain significant new information on the nature of Lp(a) especially in regard to the heterogeneity of Lp(a) and apo(a). This information will be summarized here together with some perspectives on the biochemistry and biology of this important and distinct lipoprotein class.

What is Lp(a)?

Important progress in the understanding of the nature of the Lp(a) particles has come from the development of techniques permitting the separation of these lipoprotein particles from the other plasma lipoprotein species (3,4). By density gradient ultracentrifugation, Lp(a) cannot be completely separated from the other apoB containing LDL particles because of relatively small differences in size and density. However, when additional techniques such as heparin-sepharose column chromatography or chromatofocusing are applied pure Lp(a) species uncontaminated by LDL can be obtained (7). Moreover, the studies by Fless et al. (7-9) confirmed the findings of other workers (10-12) that apo(a) is bound to $apoB_{100}$ by disulfide linkages and established that under reducing conditions apo(a) can be quantitatively removed from Lp(a) by ultracentrifugation. Apo(a) has an amino acid composition and immunological properties distinct from

that of apoB and is heavily glycosilated, 27.9% carbohydrate (9). In turn, the Lp(a-) component, i.e. an Lp(a) from which the (a) antigen has been removed, has overall physico-chemical properties similar although not identical to those of authentic LDL (i.e. a particle containing only $apoB_{100}$ at the time of isolation). Lp(a-) is comparatively larger in size and contains proportionally more triglycerides than LDL isolated from the same individual. However, Fless et al. (7,9) determined that apo(a) is in surface excess and probably loosely associated with Lp(a-). These conclusions were based on the following data: i) the random structure of apo(a) and its high degree of glycosilation (9); ii) the limited release of apo(a) but not apoB by trypsinization of Lp(a) (13); iii) the removal by trypsinization of the constraining effect exerted by apo(a) on Lp(a) as suggested by electron spin resonance studies (13); iv) the reduction of the relatively high viscosity of Lp(a) by the removal of apo(a), an apoprotein which is highly viscous (9); and v) the dissociation of apo(a) from reduced Lp(a) by centrifugation (8).

Overall, the structural information that has been gathered thus far indicates that Lp(a) is a distinct lipoprotein species and not simply an overglycosilated form of LDL. However, as is the case of other lipoproteins, Lp(a) has been found to consist of heterogeneous species differing in density, size, electrophoretic mobility, apo(a) content and chemical composition (3,4,6,7). The nature of this heterogeneity will be examined in the following section.

Lp(a) heterogeneity

The heterogeneity in density of human plasma Lp(a) is exemplified by the single spin ultracentrifugal profiles shown in Fig. 1. The profiles are those of six Lp(a) positive subjects; the shaded areas represent Lp(a). The results indicate that more than one Lp(a) species can occur within a single $Lp(a)^+$ subject; the data also indicate that there is an inter-subject variability, both in terms of Lp(a) density and concentration.

By gradient gel electrophoresis (2.5-16%) under non-denaturing conditions (i.e. absence of SDS) several species comprising Lp(a) can be resolved (Fig 2) which can be confirmed by immunoblot analyses using anti apo(a) antisera. In most instances, Lp(a) bands have a molecular weight higher than LDL. If we now use the same gradient but in the presence of SDS (Fig 3), the protein bands containing apo(a) have a molecular weight larger than apoB. Additional heterogeneity is detected when the same samples are run on SDS-gradient gels in the presence of the reducing agent 2-mercaptoethanol. In this case, apo(a) is dissociated from apoB and migrates independently from the latter. Depending upon the sample, the molecular weight of apo(a) can be higher, equal or lower than apoB (see Fig 4 for two examples). Since removal of sialic acid does not significantly affect the results it is evident that the differences in electrophoretic mobility are mostly due to actual polypeptide chain heterogeneity (unpublished observations). This apo(a) heterogeneity appears to influence the particle size of Lp(a). According to Fless et al. (7) high molecular weight apo(a) are affiliated with the high density Lp(a), apo(a) with a molecular weight in the range of apoB are affiliated with intermediate Lp(a) particles and apo(a) of a low molecular weight are affiliated with Lp(a) of a low density. Evidence recently presented by Fless et al. (9)

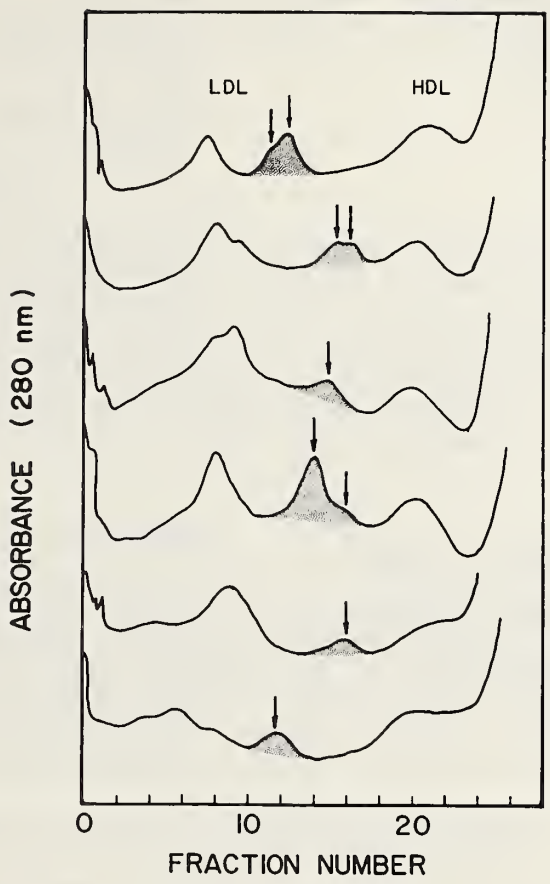

Figure 1. "Single-spin" density gradient profiles of six $Lp(a)^+$ subjects. The shaded peaks represent Lp(a). Note that several plasma lipoprotein profiles have more than one Lp(a) species.

indicates that there are two apo(a) chains per mole of apoB in a subspecies of Lp(a) called "low density Lp(a)". This observation raises the possibility that in the same particle the two apo(a) peptide chains may differ in size. Re-examination of older data suggests the further possibility that some Lp(a) heterogeneity can be caused by the presence of only one, or possibly three apo(a) species per Lp(a) particle (7). One anticipates that as more systematic studies are carried out on Lp(a) an important degree of heterogeneity will be found at the level of the carbohydrate moiety such as in the case of apoB, apoE and apoC-III.

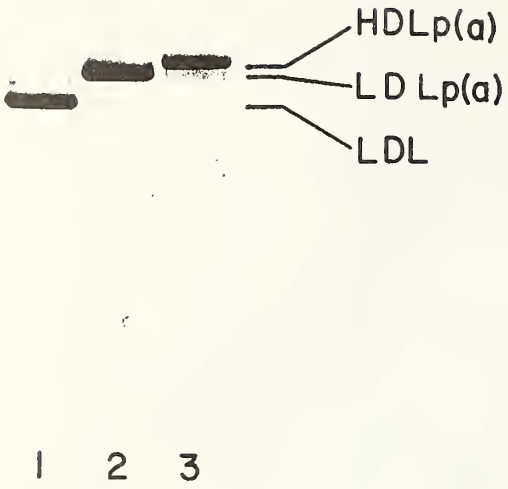

Figure 2.

Gradient gel electrophoresis of LDL (in lane 1), "low density" Lp(a) (LDLp(a), in lane 2), and "high density" Lp(a) (HDLp(a), in lane 3) in nondenaturing buffers. The three lipoproteins were isolated from the same individual and were applied to a 2.5-16% concave gradient gel (Pharmacia). The HDLp(a) fraction is contaminated with small amounts of LDLp(a).

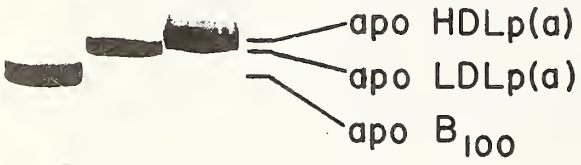

Figure 3.

SDS-gradient gel electrophoresis of LDL in lane 1, LDLp(a) in lane 2 and HDLp(a) in lane 3 on a 2.5-16% gradient gel. Lipoprotein samples are identical to those in Figure 2.

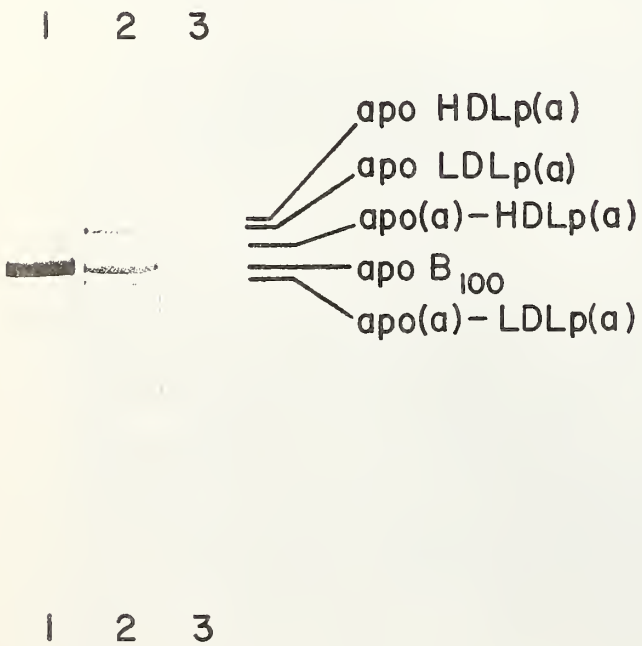

Figure 4.

SDS-gradient electrophoresis of LDL in lane 1, LDLp(a) in lane 2 and HDLp(a) in lane 3. Lipoprotein samples are identical to those of Figures 2 and 3 but where reduced with 2-mercaptoethanol. The reduction is incomplete to allow a clearer comparison of the apo(a) species in relation to the apo B-apo(a) complexes. Apo HDLp(a) is the unreduced complex of apo B-apo(a) from HDLp(a); apo LDLp(a) is the unreduced complex of apo B-apo(a) from LDLp(a); apo(a)-HDLp(a) is the free apo(a) dissociated from reduced HDLp(a); and apo(a)-LDLp(a) is the free apo(a) obtained from LDLp(a).

Implicit in the above discussion is the assumption that the apoB in Lp(a) is invariant and equivalent to the apoB present in authentic LDL. However, this may not be the case. The rapid developments in structural properties of apoB should make it possible to approach this issue with the appropriately required technology.

Methods for detecting heterogeneity

Ultracentrifugation

The single spin ultracentrifugal procedure developed in this laboratory for the separation of the various classes of plasma lipoproteins (14,15) provides a relatively simple method for identifying various Lp(a) species starting from as low as 0.2ml of plasma (we prefer EDTA plasma). This is clearly exemplified by the patterns presented in Fig 1. The advantage of the method is that samples corresponding to the Lp(a) areas can be collected and subjected to additional analyses: electrophoresis, immunoblots, etc.

Electrophoresis of native Lp(a)

Electrophoresis of plasma on 2.5 to 6% polyacrylamide gels in nondenaturing buffers also allows the detection of Lp(a) heterogeneity. Depending on the titer of the antibody and the concentration of Lp(a), between 0.1 to 10 ul of $Lp(a)^+$ plasma may be needed to detect Lp(a) by Western blotting. A representative immunoblot of 5 $Lp(a)^+$ and one $Lp(a)^-$ plasma showing size heterogeneity of Lp(a) is shown in Figure 5.

Detection of apo(a) isoforms by "Western" blotting

This represents the most straightforward method for examining apo(a) heterogeneity in whole plasma. For this purpose the plasma is separated by gradient gel electrophoresis in the presence of SDS and 2-mercaptoethanol and then subjected to the immunoblot procedure utilizing antisera specifically raised against apo(a). From the examples in Fig 6, it is evident that this technique permits the identification of apo(a) subspecies that are smaller, equal to, or larger than apo B_{100}. Analysis of this small sample of $Lp(a)^+$ individuals indicates that there are at least 5 and probably more isoforms of apo(a). The simplicity of the method makes it suitable for large scale population studies and genetic analyses.

High performance liquid chromatography.

We and others (12) have recently applied mono Q columns to the separation of Lp(a) from the other lipoprotein species. This method is also useful in separating different Lp(a) species from each other. In Figure 7a is shown the purification of high density Lp(a) obtained from a 7.5% CsCl self generating gradient (50.2 Ti rotor, 49,000 rpm, 20°C, 20h). When this fraction was applied to the mono Q column partial separation of the two Lp(a) species was obtained (Figure 7b). These preliminary results are encouraging but more work remains to be done to further refine this technology.

1 2 3 4 5 6

Figure 5. Identification of plasma Lp(a) by gradient gel electrophoresis and "Western" immunoblotting. Five microliters of plasma from five Lp(a)+ and one Lp(a)- individual (lane 4) were applied to a 2.5 to 6% linear polyacrylamide gradient gel. The primary antibody was rabbit antihuman apo(a) and the secondary antibody was goat antirabbit IgG labelled with horseradish peroxidase.

1 2 3 4

Figure 6. Identification of apo(a) isoforms present in the plasma of four Lp(a)+ individuals by SDS gradient gel electrophoresis and "Western" immunoblotting. One microliter of plasma was treated with SDS and 2-mercaptoethanol and applied to a 2.5-16% gradient gel (Pharmacia). Immunoblotting was carried out as in Figure 5. The arrow on the left identifies the position of apo B_{100}. Five isoforms were detected; three (a-c) with a mobility less than apo B_{100}, and one each with a mobility equal to (d) and greater (e) than apo B_{100}.

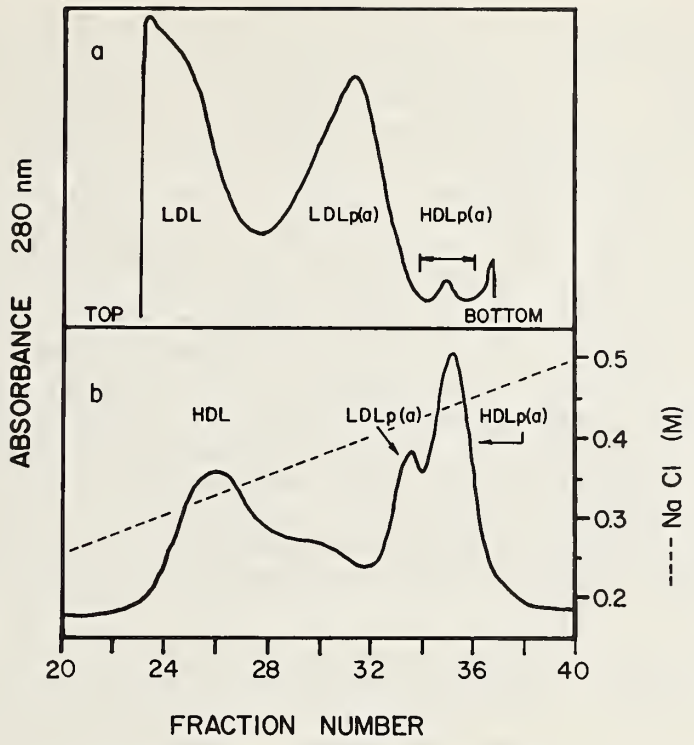

Figure 7. Separation of Lp(a) species by HPLC-ion exchange chromatography.

a: Fractionation of Lp(a) by density gradient centrifugation on a 7.5% CsCl self generating gradient run in the 50.2 Ti rotor at 49,000 rpm at 20°C for 20h. The HDLp(a) fractions indicated by the arrows were pooled and taken for further purification by HPLC.

b. HPLC-ion exchange chromatography of HDLp(a) on a mono Q column (Pharmacia). HDLp(a) was dialyzed against 0.010 M TRIS/HCl, pH 7.4 before application to the column. Sample load was approximately 1 mg but can be increased to 10 mg. It was eluted with a 40 minute gradient from 0 to 1 M NaCl superimposed on 0.010 M TRIS, pH 7.4 at a flow rate of 1 ml/min at room temperature.

Anti apo(a) affinity columns.

Several monoclonal antibodies against apo(a) have been developed. It is possible that they may exhibit a preferential reactivity for given isoforms of apo(a). In such a case these monoclonals can be covalently linked to a solid support and serve as useful markers for apo(a) heterogeneity, or as reagents in determining the abundance of different Lp(a) species in plasma.

Physiology of Lp(a)

Little knowledge is available on the tissue of origin of Lp(a), on its mode of synthesis, secretion and degradation. Although the liver may be a source of Lp(a), at this time the evidence to support this statement is weak. In this laboratory we have been unable to demonstrate immunologically reactive Lp(a) in the medium of Hep G2 cells or in liver perfusates of rhesus monkeys, animals which are known to contain Lp(a) in their plasma (16). However, it is possible that the experimental conditions followed were not ideal for the identification of these lipoprotein particles. A suggestion that Lp(a) may originate from the intestine has come from the studies of Bersot et al. (17) showing that human subjects fed a fat rich diet have plasma chylomicron remnants with a component reacting with antibodies specific for Lp(a). By gradient gel electrophoresis, the post-prandial apo(a) was found to have molecular weights higher than the two apo(a)s associated with plasma Lp(a). However, the data are puzzling in that apo(a) was found to be linked to B_{100} when the intestine produces mainly B48. Furthermore, it is surprising that apo(a) is produced by the intestine although it is known that the levels of Lp(a) are neither significantly affected by diets (2-4) or by fasting (18).

Germane to the issue of Lp(a) synthesis is that concerning the origin of apoB and apo(a). Based on the lack of correlation between the levels of apoB and apo(a) in the plasma one may postulate that the synthesis of these two apolipoproteins is not coordinated. However, the possibility can be entertained that the plasma levels of apoB and apo(a) are influenced by their rate of proteolytic degradation, apo(a) being more susceptible to proteolysis than apoB. It is of interest to note that in our experience the plasma levels of apoB always exceed those of apo(a); in turn, the patients with abetalipoproteinemia examined in this laboratory have shown to have neither Lp(a) nor apo(a) in their plasma (unpublished results). Whether this indicates a dependency of the apo(a) synthesis on apoB remains to be established. Of interest in this regard is the study by Malloy et al. (19) who discovered a patient with normotriglyceridemic abetalipoproteinemia having only small amounts of B48 in the plasma. When this plasma was tested against antiserum said to be specific for Lp(a) a precipitin arc in immunodiffusion plates was observed. If confirmed, these results are of interest in that all studies reported thus far have indicated the occurrence of chemical linkage of apo(a) and with $apoB_{100}$ but not with $apoB_{48}$. However, it is also possible that in the patient apo(a) is not associated with apoB, a question currently examined in this laboratory.

The site of linkage between apoB and apo(a) is also unresolved. Although an intracellular event can be postulated, lipoprotein-free apo(a) has been detected in the plasma and this raises the possibility that apo(a) may bind to LDL by an extracellular process. Based on the current uncertainties on the site and mode of origin of Lp(a) it is not surprising that little is known on the physiological role of this particle, if any. Rather, the presence of Lp(a) in the plasma has been associated with pathology. This evidence is discussed below.

Genetics of Lp(a)

Based on his classification of $Lp(a)^+$ and $Lp(a)^-$ subjects, Berg (1,2) has proposed that the Lp(a) antigen is governed by an autosomal dominant mode of inheritance and the formal genetic analyses of the data support this conclusion (4). The Lp(a) antigen has been observed in all populations studied with an incidence ranging from 2-35% and not less than 20% of the Swedish population (2). From a complex segregation analysis of 229 nuclear families, Iselius et al. (20) have concluded that Lp(a) is a dominant gene with a frequency of 0.10 and penetrance of 0.92. Neglegible evidence has been found for an environmental effect on the levels of plasma Lp(a) indicating a high degree heritability. The analyses of multiple data sets have suggested a major locus with three alleles, Lp^A, Lp^a and Lp^o determining the high levels of Lp(a) in plasma (21). Moreover, Morton et al. (22) have proposed that an additional recessive gene is responsible for the low levels of plasma Lp(a). Early linkage analyses have shown an association between Lp(a) levels and red cell esterase and this observation has led to suggest that the apo(a) gene may be located in chromosome 13 (2). In turn, no association has been found between Lp(a) and Ag genetic markers. Recently the studies by Guyton et al. (23) have indicated that the incidence of Lp(a) in black men (31.3 + 2.8) and women (33.7 + 2.3) is approximately twice as high as that of white men (17.0 + 2.3) and women (15.5 + 2.2). The basis for this racial difference was not determined.

Lp(a) as an atherogenic factor

Several studies have shown that the levels of Lp(a) in the plasma are positively correlated with coronary heart disease (23-26). In a series of patients undergoing corrective surgery for occlusive peripheral vascular disease we found that about 60% of them had plasma levels of Lp(a) cholesterol above 30mg/dl (unpublished observations). Since in many of these cases the Lp(a) elevation was not associated with other detectable forms of hyperlipidemias the data appear to corroborate the view that Lp(a) represents an independent risk factor for these types of patients. In addition, in the Lipid Clinic at the University of Chicago, particular attention is being paid to those patients who present with normal or borderline normal plasma cholesterol values but with personal and/or familial history of cardiovascular disease. A good percentage of these patients prove to be $Lp(a)^+$. The reasons for the apparent atherogenicity of Lp(a) have not been established. We may speculate that this may relate to the ability of Lp(a) to strongly interact with the glycosaminoglycans of the arterial wall as well as with calcium ions (3). In this context it may be noted that Lp(a) has been detected immunologically in arterial tissues (27). An intriguing possibility is that Lp(a) traverse readily the endothelial cells leading to an increased influx of these particles into the arterial wall and accumulation within it. Granted that Lp(a) is an atherogenic factor, means must be devised to decrease its plasma levels. Our experience thus far corroborates previous findings that diets have little effect in this direction. Rather promising appear our observations that $Lp(a)^+$ patients with heterozygous familial hypercholesterolemia exhibit a 20-30% reduction of plasma Lp(a) during treatment with cholestyramine and nicotinic acid (unpublished observations). Moreover, the recent findings that treatment of type II hyperlipoproteinemic subjects

with a combination of neomycin and niacin lowers their plasma Lp(a) levels is important in this regard (28). Additionally, the anabolic steroid stanazolol has been shown to lower the concentration of Lp(a) in plasma (29).

Concluding remarks and perspectives

About 25 years from its discovery we now know that Lp(a) consists of an LDL-like particle, Lp(a-) containing apoB100 linked by disulfide bridges probably to two apo(a) molecules which are chemically and immunologically distinct from apoB and comparatively richer in carbohydrate. The Lp(a) is heterogeneous in size and density and such heterogeneity involves mainly apo(a) but the $Lp(a)^-$ portion may also contribute to it. Various molecular forms of apo(a) have been described but their structural relationship and function are yet unknown. Lp(a) is a genetically-determined particle with an apparent autosomal dominant mode of inheritance and is described in the plasma of all populations studied.

In spite of much progress, little is known on the site and mode of synthesis of this lipoprotein variant as well as on its secretion and degradation. According to current studies, apo(a) can either occur lipoprotein-free or as a part of the Lp(a) complex; however, the relationship between these two different forms remains undetermined. Where and when apoB and apo(a) link to each other is unknown and this process may involve both extra- and intra-cellular events. It is indeed puzzling that only some LDL species are destined to associate with apo(a), an apolipoprotein for which a tissue source has not yet been established. The rapid progress made in the area of apoB, particularly in the isolation of cDNA clones (30-34) should facilitate the investigation of Lp(a) both on the structural and functional levels. From what we understand today the presence of this particle in the plasma correlates with a high incidence of cardiovascular disease. In this context, the problem of the apo(a) polymorphism deserves a special attention in that the pathology related to Lp(a) may also relate to the prevelence of a given apo(a) isoform besides its overall lipoprotein mass. Our methods of detection and analysis of these isoforms are in need of refinement and simplification so that they can be applied on a large scale. As work progresses valuable reagents such as monoclonal antibodies, cDNA probes and derivatives thereof will become available and they should stimulate additional interest and research directives. As it now stands, it is legitimate to ask whether apo(a) deserves to be classified as an apolipoprotein. This uncertainty, however, even if justified, should not prevent continuing studies on this interesting biomolecule still in search of function.

Acknowledgments

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SESSION III. DISCUSSION OF HETEROGENEITY OF CHOLESTERLY ESTER RICH LIPOPROTEINS ASSESSED IN VITRO

DR. BARTER: Dr. Weisgraber, in relation to your comment about the mechanism of formation of HDL-C and HDL-1, some work that we published a couple of years ago strongly supports that mechanism in the rat, which is a lipid transfer protein-deficient animal and very high in activity of HDL-1 and susceptible to HDL-C. When we injected human transfer protein in vivo into these animals, the HDL-1 disappeared and the ApoE disappeared. We think that this result occurred not so much because of an increased uptake by tissues, but due to a diminished formation of HDL-1 in these animals. This explanation would fit in exactly with your hypothesis.

DR. WEISGRABER: I'm aware of those studies and I agree that my hypothesis is consistent with what you are saying.

DR. PATERNITI: What troubles me about postulating that ApoE-rich HDL-C is a major carrier and is metabolically important for cholesteryl transport is that we haven't got any notion of what the actual flux of cholesterol through that compartment is. There doesn't seem to be a lot of it in plasma and we don't have any good evidence that the bulk of cholesterol transport ever winds up in that particle. It is one thing to postulate that cholesterol can be removed by interaction with HDL with an E receptor and it is another thing to rationalize how significant that is.

DR. WEISGRABER: I agree. In human plasma, it probably is present in low concentration if there at all, but in the dog that is not true. So, it depends on the species that you are dealing with. That is the point that I made and Dr. Barter has made about the cholesteryl ester transfer activity. It depends on the species you are looking at.

DR. SCANU: Dr. Weisgraber, I am interested in your comment about the complex A2: ApoE influencing the distribution of ApoE among HDL and VLDL. We always have a difficult time finding bona fide HDL-C in the rhesus monkey. The rhesus monkey AII is a monomer so in that case you cannot have a complex between ApoE and ApoAII.

DR. WEISGRABER: That would be similar with the E4/E4 patients. You get a distribution toward VLDL rather than HDL-C.

DR. WITZTUM: Dr. Weisgraber, do you have any evidence that there are complexes with ApoE with ApoB and, if not, why not?

DR. WEISGRABER: We looked for that and have not been able to see any of those complexes and I don't know why.

DR. GIANTURCO: Dr. Weisgraber, it is very interesting that the conformation of ApoE in the HDL is what you see in the other. Have you had any opportunity to look at the conformation in the large, very slow lipoproteins because we see things comparable. ApoE, we can assume is different there because the VLDL of type three subjects have K_D similar to binding in E to the LDL receptor as those with E3 or E4.

DR. WEISGRABER: Your studies show quite nicely that there is a conformational effect on these. It was hard enough to do the HDL particles and we haven't tackled the others yet.

DR. FOGELMAN: Dr. Innerarity, would you please comment on any differences between rabbit beta VLDL and canine beta VLDL? All of these studies were beta VLDL, is that correct?

DR. INNERARITY: What Dr. Fogelman is referring to is a joint study we did in which we looked at the degradation of canine beta VLDL and rabbit VLDL with his original FH patient. We saw appreciable amounts of degradation of the rabbit beta VLDL but very little degradation of the canine beta VLDL. Certainly there can be another process, but when we've looked for cholesterol esterification using his rabbit beta VLDL we have not found any in the FH monocyte macrophages. So there may be another process that is degrading the rabbit beta VLDL, but not producing cholesteryl ester formation. Would you like to comment on that. I don't know if you've ever done that with the FH patient.

DR. FOGELMAN: No, we didn't look at cholesterol Esterification, but the uptake and degradation were markedly different. As you alluded to in the studies you did with Brian Milantin, the uptake to rabbit beta VLDL in both normal and in the FH patients monocyte macrophages were about ten-fold higher with rabbit beta VLDL than canine beta VLDL. Also, canine beta VLDL acted just like LDL in the normal monocyte macrophages as well as in that child's monocyte macrophages. In terms of esterification, we have not looked in the child. So, I can't comment.

DR. INNERARITY: Well, that would be worthwhile doing because the key to this is whether this is the receptor that mediates foam cell formation or not.

DR. GIANTURCO: We all believe that there are LDL receptors on the macrophages and that they certainly can mediate the uptake of beta VLDL. Dr. Innerarity, were your studies with the human monocyte macrophages done under conditions to up regulate the LDL receptor? I ask this question because in the studies that we've done with macrophages, we have evidence that there is a distinct sector that does not require ApoE and it is not up-regulated under the conditions that up-regulate the LDL receptor. In fact, it can sometimes suffer from the up-regulation of the LDL receptor and it actually goes down. The second question is whether or not you've had the opportunity to look at any VLDL from hypertriglyceridemic humans with these cells from the FH homozygote to see whether they enter the cells. Of course, if the LDL receptor is present and the appropriate E is there, they can bind and we also see that. We also see another receptor that doesn't require E. So, first, are the human monocytes up-regulated and, second, have you looked at the humans?

DR. INNERARITY: Yes, the receptors are up-regulated in human studies.

DR. GIANTURCO: So, possibly to other receptor could be examined.

DR. INNERARITY: It is certainly possible and I don't disagree at all that there can be another receptor that can bind to these lipoproteins once they have been treated with certain proteases. It's just that we see the native lipoproteins or at least the vast majority of it appears to be attributable to the LDL receptors. When we've treated some lipoproteins with trypsin, we've also seen an increase in the uptake by another process that wasn't the LDL receptor. I don't know if we're still in disagreement, extrapolating back from the protease-treated lipoproteins, to say that the same thing happens with the native lipoproteins may be probably the only area that we still disagree on.

DR. GIANTURCO: We do find VLDL from certain hypertriglyceridemic subjects that have evidence of ApoB breakdown and these and only these VLDL can load macrophages. So we feel that there is a correlation. We've presented results elsewhere that show that prothrombin and thrombin do associate with VLDL and can process ApoB.

DR. INNERARITY: I think now we're clarifying the differences that we had. In your case, it is more of a receptor that has seen lipoproteins that have been processed by thrombin.

DR. GIANTURCO: That's correct.

DR. SCHONFELD: Dr. Innerarity, as I understood your experiment, you were comparing human fibroblasts and mice macrophages and human monocyte-derived macrophages. When you compared the human to the human, the fibroblasts and the macrophages are regulated about the same way. Is that correct?

DR. INNERARITY: Right.

DR. SCHONFELD: But when you are comparing the mouse cell to the human cell, the mouse cell seems to have a defective kind of regulation.

DR. INNERARITY: That is correct.

DR. SCHONFELD: Is that related to the macrophage or to the mouse? Have you looked at mouse fibroblasts?

DR. INNERARITY: Yes, we have. It appears it is not real clear-cut, but with a mouse fibroblast they are regulated the same way that mouse macrophages are. There appears to be some mouse cell lines, like the Y1 adrenal cells, that have somewhat different properties, but in several fibroblast cell lines from mice that we've looked at, the properties are very similar to the LDL receptor on mouse macrophages. The answer is it's species specific for the most part, but that may not be the whole story.

DR. SCHONFELD: That makes a big difference because it would have an altogether different implication.

DR. GINSBERG: Dr. Innerarity, following up on species again, I wasn't sure of your answer to Dr. Gianturco's question. Patsy Wong-Iverson and I showed in a single FH patient that normal human VLDL was degraded in a normal fashion by that patient's monocyte-derived macrophages where LDL degradation was less than one percent of control. I'm not sure if I understood you as to whether or not you looked at human VLDL in this system?

DR. INNERARITY: No, we haven't but there is one other factor that we should discuss. Brown and Goldstein's lab have now created a number of site-specific mutagens in the ligand-binding region of the LDL receptor and found that almost any type of mutation that they make destroys the LDL binding, but the ApoE-containing lipoproteins combine normally and they've actually seen some of these in FH receptor-negative cells now. So, it is certainly possible that you could have been looking at one that would still bind ApoE-containing lipoproteins normally, but very defective in LDL binding.

DR. BARTER: To pursue the species question a little bit further, is it possible that the reason that your mouse macrophages don't recognize human LDL is because it's human LDL? What about mouse LDL against mouse macrophage? Do they appear to have LDL receptor activity when you use that type of system?

DR. INNERARITY: Mouse LDL and human LDL behave exactly the same.

SPEAKER: Dr. Scanu, have you used monoclonal antibodies to characterize the subspecies of the Lp(a)? Could you comment on, for example, Gerd Utterman's work?

DR. SCANU: We used a polyclonal antibody from Linda Curtiss and all her monoclonal antibodies to B100 did not react. Our polyclonals don't cross-react. We've been actually aiming at finding something that could see the linkage, the disulfide bond, but thus far we have failed.

DR. KRAUSS: Dr. Scanu, my question concerns the size heterogeneity of the Lp(a) which we've also seen when we've done electropheresis. We've had the sense that there is some relationship of the major size of the Lp(a) species and the category of LDL that is present in the plasma. Do you suppose the size of the ambient LDL is at all related to where the Lp(a) is on your spectrum?

DR. SCANU: We have not looked systematically at this issue. We are interested in defining the size in general, but that is a very good issue. In fact, the overall relation between Lp(a) in normal subjects and in hyperlipidemic subjects in something that we need to look at in much more detail.

DR. BABIAK: We work with non-human primates, rhesus monkeys, that also show the Lp(a) phenotype and we've seen in general something that Dr. Krauss has referred to, that there is a relationship to the LDL size and the Lp(a) size. In fact, in some brief experiments we reacted the Lp(a) with mercaptoethanol and ran that side by side with the native LDL of the animal and they lined up just exactly right on the gradient gel. If their Lp(a) is larger, their LDL is larger.

DR. SCANU: We are also looking at rhesus monkeys which are much more interesting from a genetic aspect because in a family of monkeys with Lp(a) we can look at the isoforms. The only problem is that the phenotype of Apo(a) has exactly the same molecular weight as B100. I think the question is how frequent is Lp(a)? Is there an Lp negative versus pseudo-Lp negative? It is still unsettled because we are finding some false reactivity, something called Lp(a) positive. If you start testing immunoblots and molecular weight you can see reactions against some of the antibodies with a much lower molecular weight, particularly species that are not affiliated with the lipoproteins, but are in bottom fraction.

DR. GINSBERG: Dr. Scanu, if you take the (a) protein and incubate it back with Lp(a) minus, does it reassociate and, if so, does it associate with other subclasses of LDL at difference sizes and density that weren't Lp(a) positive?

DR. SCANU: Thus far we have not been able successfully to do such a thing, but we are trying.

DR. FLEISH: I'd like to reply to Dr. Babiak's question. We looked at the Lp(a) and the LDL from an autologous situation and the LDL was always smaller than the Lp(a) minus and I just have a feeling that you can't pick up the difference using a gradient gel.

DR. BARTER: I just caution interpreting molecular weight from the gradient gels because, if your hypothesis of this being something added on to the outside, you're going to alter the shape of the particle and that actually has quite a big effect on

how far it migrates on a gradient gel. So, to try to get this concordance between what you predict the molecular weight of the particle to be plus or minus the tail, you're going to have all sorts of problems.

DR. FLEISH: We didn't determine the molecular weight by gradient gel electropheresis. This was done by analytical centrifugation which isn't affected by shape.

SESSION IV. Metabolism *In Vivo* of ApoB-Containing Lipoproteins

Chair: Scott Grundy

THE METABOLISM OF LARGE AND SMALL VERY LOW DENSITY LIPOPROTEINS IN NORMAL AND DYSLIPOPROTEINEMIC STATES

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Introduction

Plasma very low density lipoproteins (VLDL) are an heterogeneous population of particles isolated in the density interval 0.95-1.006 g/ml (Sf 20-400). The largest, least dense VLDL are rich in triglyceride while smaller species have increased contents of cholesterol and protein. Apolipoprotein B is the major protein constituent of VLDL. When labeled with iodine it acts as a useful tracer of the particle's metabolism.

Normal individuals given a bolus injection of autologous ^{125}I-VLDL transfer more than half of the labeled apo B into low density lipoprotein (d 1.019-1.063 g/ml; Sf 0-12) via an intermediate species (IDL, d 1.006-1.019 g/ml; Sf 12-20) (1,2). That is, a precursor-product relationship exists between VLDL and denser apo B containing lipoproteins. In dyslipoproteinemic states, however this link is broken and other novel routes of apo B synthesis and catabolism appear (3,4). These conclusions drawn from early metabolic studies are based on the thesis that VLDL and its catabolic products can be viewed as homogeneous entities whose behavior within a given density interval is uniform. However, recent structural investigations of VLDL, IDL and LDL reveal the presence of multiple species which may exhibit individual rates of formation and breakdown. VLDL, for example, contains particles of varying lipid and apoprotein compositions. Some, the smaller species, are readily assimilated by cells via receptor mediated processes while larger VLDL are resistant to these effects (5). On the other hand, lipid exchange interactions seem to occur more readily with large triglyceride-rich VLDL (6). It is clear then that numerous mechanisms other than simple triglyceride hydrolysis are responsible for the remodelling and degradation of VLDL and that to begin to understand these we must dissect the apo B containing lipoproteins into relatively homogeneous sub-populations whose metabolic properties can be defined with some certainty. Since it is not yet clear what the basis of such a separation technique should be, we have as a first approximation used cumulative flotation ultracentrifugation (7) to separate lipoproteins on the basis of density and size. This procedure has been used to follow the metabolic properties of large (Sf 60-400) and small (Sf 20-60) VLDL in normal and hyperlipidemic states.

Cell culture studies have shown that hepatocytes can synthesise and secrete particles covering a broad size range (8), and in vivo it has been observed that apo B is inserted into VLDL throughout the Sf 20-400 distribution (9). The key question raised by Fisher (10) is whether such heterogeneous nascent particles ever attain metabolic equilibrium in the circulation, or alternatively are they catabolised via different metabolic channels depending on their origin? In a preliminary series of investigations (Study 1, Table 1) the transit of B protein was followed from large VLDL through its smaller delipidation products to LDL. Little of the radioactivity which was originally associated with large VLDL reached LDL. In light of that, the Sf 20-400 VLDL spectrum was fractionated in an attempt to define more clearly the origins and fates of its constituent particles.

Table 1 APOLIPOPROTEIN B METABOLISM

EXPERIMENTAL PROTOCOL

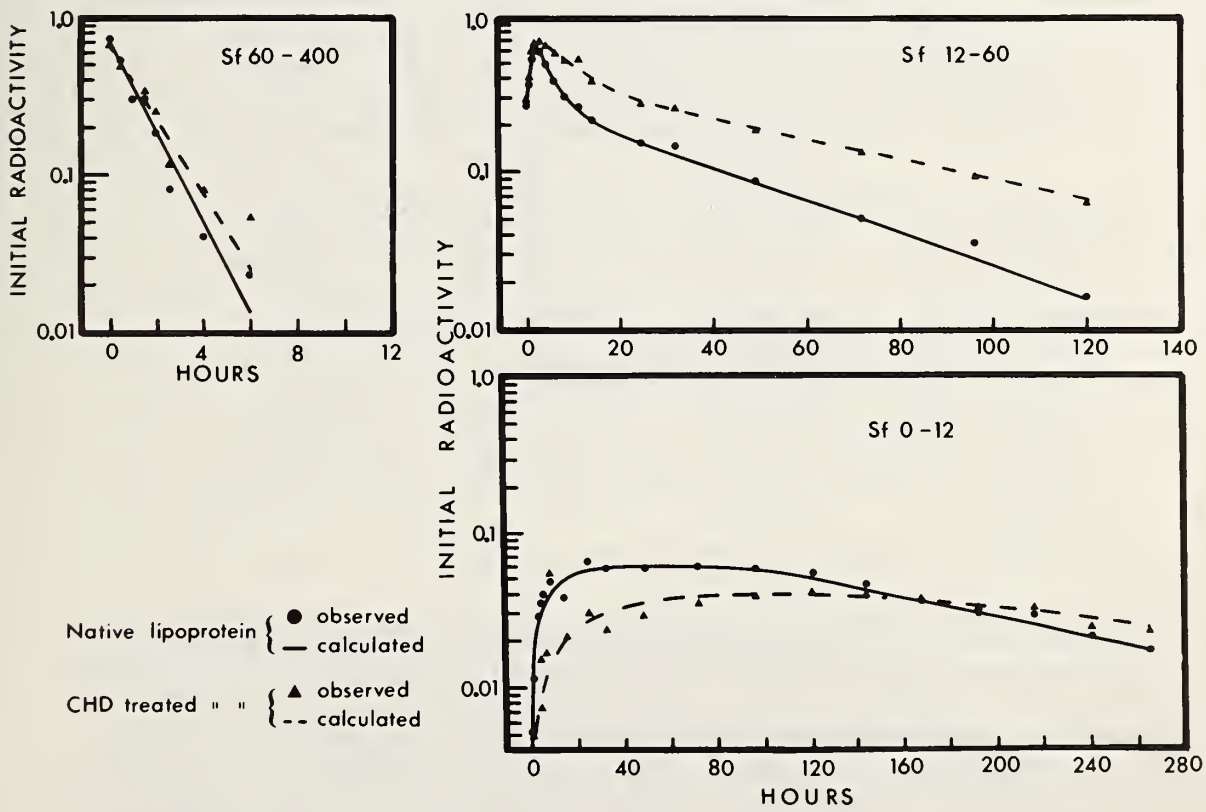
Study	Subjects	Sf 400 60 20 12 0
1	Normal n = 6	^{125}I
		$^{125}I + ^{131}I$ CHD
2	HTG ★ n = 6	^{125}I
3	DYS β ★ n = 6	^{125}I
		^{131}I
4	FH hetero ✱ n = 6	$^{125}I + ^{131}I$ CHD
5	FH homo n = 7	^{125}I
		^{131}I

★ ± bezafibrate
✱ ± cholestyramine

Within a few hours of injection of ^{125}I-labeled Sf 60-400 VLDL into normolipemic subjects, the tracer transferred virtually quantitatively to the Sf 12-60 flotation interval. Thereafter, however, it failed to progress into Sf 0-12 LDL (11). We concluded on the basis of this observation that Sf 12-60 remnants of VLDL lipolysis must be subject to direct clearance from the circulation, possibly by a process which involves the agency of cell membrane receptors. This hypothesis was tested by modifying the arginine residues on the B protein of the VLDL tracer in order to interfere with any potential receptor interaction.

The modification did not change the rate at which large triglyceride rich particles were converted to smaller remnants (Figure 1), a metabolic step which is thought to depend on lipoprotein lipase activity, but it did retard the clearance of the Sf 12-60 particle from the circulation (12). So, receptors do seem to have an involvement in remnant catabolism. Some of the B protein in the tracer (about 10%) appeared in LDL, but at a rate which was also delayed by arginine modification. This raises the question of another, possibly separate role for receptors in mediating the conversion of Sf 12-60 remnants of VLDL metabolism to LDL.

Figure 1 Transit of native and 1,2 cyclohexanedione-modified apo B from VLDL (Sf 100-400) through IDL (Sf 12-100) to LDL (Sf 0-12)



Clearly, most LDL cannot originate from large VLDL but presumably is derived from smaller particles of Sf 20-60. When these were labeled directly, the majority transferred into LDL (11). So, the Sf 12-60 density range contains a mixture of lipoproteins only some of which are destined to make LDL and within this interval there are particles whose fate depends on their pedigree; that is, there appears to be metabolic channelling within the VLDL flotation interval.

VLDL metabolism in hypertriglyceridemia

The protocol devised to examine B protein metabolism in Study 1 was extended to hypertriglyceridemic individuals (Study 2, Table 1). In this group, large VLDL was catabolised more slowly than normal, with a consequent increase in its plasma concentration. Bezafibrate corrected the defect and reduced the VLDL pool size by 70% (13). Interestingly, the drug did not alter the rate of catabolism of the remnants generated from these large triglyceride-rich particles, although it did diminish the catabolic rate of LDL and expand its circulating mass (Table 2).

Table 2 EFFECTS OF BEZAFIBRATE ON APOLIPOPROTEIN B METABOLISM IN HYPERTRIGLYCERIDEMIA*

	Fractional Catabolic Rate (pools/d)		
	Sf 100-400	Sf 12-100	Sf 0-12
Control	7.0±7.5	1.23±0.55	0.47±0.25
Bezafibrate	22.9±24.0	0.98±0.38	0.35±0.12
paired t test	<0.05	NS	<0.05

* n=6

It is apparent therefore that fibrates can exert various effects at different points on the Sf 0-400 metabolic cascade. Their known stimulatory influence on lipoprotein lipase accords with the suggestion (see above) that this enzyme governs the clearance rate of large triglyceride rich VLDL. But the lack of effect on the rate of Sf 12-60 remnant catabolism, a process which in fact was not abnormal in the hypertriglyceridemic individuals indicates that lipase is not critical in their catabolism. Presumably their removal was mediated by "receptors" as suggested for the normolipemic group examined in Study 1.

The response of LDL to fibrate therapy was investigated in greater detail in another group of hypertriglyceridemic volunteers. It transpired that the drug suppressed the hyperactivity of catabolic processes operating independently of the LDL receptor which apparently were responsible for the low circulating LDL levels in this group (14). As a result, during treatment, more LDL became channelled into the receptor route.

VLDL metabolism in dysbetalipoproteinemia

Apolipoprotein E (Apo E) exists in three major isoforms, designated E_2, E_3 and E_4. Apo E_3, the wild type protein, is common in the population, while the rarer E_2 and E_4 appear to have arisen by point mutations (15). Recent studies have shown that the isoforms exert an influence on B protein metabolism in that subjects homozygous for E_4 have higher plasma LDL levels while in E_2 homozygotes this parameter is reduced (16). When an as yet unknown second stimulus is applied to the latter group, frank Type III hyperlipoproteinemia ensues (15), with characteristic compositional anomalies in VLDL and accumulation of IDL in the plasma. Previous investigations have shown that the conversion of VLDL through IDL to LDL is slower in these individuals (17). We set out to reinvestigate the problem further as outlined in Table 1, Study 3. Large and small VLDL of Sf 60-400 and 20-60 were radiolabeled and their metabolic fate followed in six subjects before and during bezafibrate therapy (18). The rate of catabolism of large VLDL was slow in these subjects and, as noted for other hypertriglyceridemic subjects (Table 2) accelerated during fibrate therapy. This effect, coupled with the reduction in B protein synthesis which accompanied administration of the drug, led to an 80% decrement in the circulating level of Sf 60-400 apolipoprotein B (Table 3).

Treatment also reduced the input of apo B into small VLDL but did not increase the characteristically slow rate of catabolism of these particles in the Type II subjects. So again, bezafibrate had diverse effects at different points in the metabolic cascade. Interestingly, although treatment, reduced LDL catabolism by up to 50%, as it had done in the hypertriglyceridemic subjects (Table 2), we did not record a rise in the plasma concentration of the fraction. This was because therapy had simultaneously suppressed LDL synthesis by promoting direct clearance of IDL from the circulation. Such an effect might be due to increased hepatic B/E receptor activity.

Apolipoprotein B metabolism in Familial hypercholesterolemia

Familial hypercholesterolemia (FH) derives from defective expression of the LDL receptor on cell membranes and, in consequence, there is failure to clear the lipoprotein normally from the circulation. Not only does this lead to an increase in the level of LDL in the plasma but there are also increments in VLDL and IDL (19). Studies 1-3 (Table 1) provide evidence that the B/E receptor appears to be involved in VLDL and IDL metabolism as well as that of LDL. Two additional investigations (Studies 4 and 5) were designed to follow the metabolic consequences of partial or complete receptor deficiency. FH heterozygotes, who express only half of the normal LDL receptor complement have high circulating levels of intermediate (Sf 12-60) lipoproteins, partly because they can convert them only slowly to LDL. Treatment with cholestyramine reduced their plasma concentration by promoting their catabolism.

In a more detailed assessment the metabolism of large and small VLDL apo B was examined in a group of 7 subjects homozygous for the FH defect. Here, large VLDL underwent normal conversion to smaller remnants which accumulated in the circulation because their subsequent turnover was defective. Two distinct patterns of metabolic behavior characterised the smaller VLDL fraction. Some subjects converted VLDL

Table 3 THE EFFECTS OF BEZAFIBRATE ON APOLIPOPROTEIN B METABOLISM IN LARGE AND SMALL VLDL OF TYPE III HYPERLIPOPROTEINEMIC SUBJECTS*

	Apo B in large VLDL Sf 60-400			Apo B in small VLDL Sf 20-60		
	Synthetic rate ($mg.kg^{-1}.d^{-1}$)	Plasma concentration (mg/dl)	Fractional catabolic rate (pools/d)	Synthetic rate ($mg.kg^{-1}.d^{-1}$)	Plasma concentration (mg/dl)	Fractional catabolic rate (pools/d)
Control	10.8±3.0	15.5±5.9	2.0±0.9	13.8±1.2	30.8±8.1	1.2±0.3
Bezafibrate	4.7±0.9	2.9±0.9	4.5±1.4	9.7±2.1	14.6±2.5	1.66±0.35
paired t test	<0.005	<0.01	<0.05	<0.01	<0.02	NS

* n=6

apo B through IDL to LDL at a very slow rate but in sufficient quantity to account for all apo LDL synthesis. In others there was rapid transmission of some VLDL apo B radioactivity directly into LDL but this pathway contributed little to the LDL B protein mass. It was therefore necessary to postulate a direct influx of apolipoprotein B into the IDL or LDL density interval in such individuals. So, receptor deficiency seems to be associated with (a) reduced direct catabolism of remnants derived from large VLDL; (b) slow B protein transit from VLDL though IDL to LDL; (c) prolonged residence of LDL in the circulation. The severe impairment of IDL to LDL conversion in these subjects provides strong supportive evidence that the receptor might be involved in this process.

Conclusions

The kinetic studies described above indicate that VLDL (Sf 20-400) can be viewed as containing at least three separate entities. Large triglyceride rich VLDL are found at the upper (least dense) end of the spectrum. The formation of these is probably favored in carbohydrate feeding and familial hypertriglyceridemia. The delipidation of these large VLDL mainly by the action of lipoprotein lipase results in the formation of remnants in the Sf 12-60 density interval. It is possible that the size of the remnant formed depends on the activity of lipase. If this enzyme is functioning normally then the residence time of VLDL is short and there is only a limited opportunity for cholesteryl ester to be incorporated by exchange from HDL into the VLDL core (20). Reduced lipase activity, in contrast prolongs the circulation time of large VLDL and favors the enrichment of the particle in cholesteryl esters. This then results in larger remnants in the VLDL density range. Little of this apo B transits the delipidation cascade to LDL and in fact it appears to have a metabolism analogous to that of gut-derived chylomicrons ie the formation of nascent triglyceride-rich particles which are lipolysed to remnants and cleared directly from the plasma via receptor-mediated pathways. Most LDL comes from small VLDL secreted into the Sf 20-60 density interval. The initial catabolic rate of these particles is slower than that of the larger species and from the results of type III and FH studies appears to involve both the 'B/E' (LDL) receptor and apolipoprotein E. In one extreme case of homozygous FH we observed that the conversion of small VLDL, through IDL to LDL took 9 days to complete. There is evidence to suggest that the formation of small VLDL is favored in familial combined hyperlipidemia and in dietary cholesterol supplementation and this may to a degree explain the increased levels of LDL associated with these conditions.

The further analysis of VLDL metabolism requires that methods are devised to permit the separation of metabolically distinct species that are at present co-isolated by size or density fractionation. One likely approach will be to prepare subfractions of differing apoprotein content by immunoaffinity chromatography and test their metabolic properties both in vitro and in vivo.

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THE ROLE OF APO E IN THE METABOLISM OF PARTICLES CONTAINING APO B

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Two mammalian apolipoproteins, apo B-100 and apo E, possess determinants recognized by lipoprotein receptors that mediate endocytosis and lysosomal catabolism (1). In each case, current evidence suggests that each protein has only a single such determinant. The binding domain of apo E has been extensively characterized by Weisgraber and his associates (2). The amino acid sequence of apo B-100 has recently been determined (3,4); its binding domain seems to be located in the C-terminal one-half of the protein, consistent with absence of this domain in the B apoprotein of chylomicrons (apo B-48), which includes determinants contained in the N-terminal region of the B-100 sequence (5).

Apo B-100 exists as a single copy in those lipoproteins that contain it (VLDL, IDL, LDL, and the Lp(a) lipoprotein). By contrast, the number of apo E molecules in these particles varies from none to as much as 20 or more (6-8). As discussed elsewhere (8-11), the number of copies of apo E on these lipoprotein particles in which the receptor-binding domain is suitably exposed evidently has a profound influence on their metabolic fate. Particles with several molecules of apo E tend to bind to lipoprotein receptors with high affinity and to be removed rapidly from the blood by receptor-mediated endocytosis, whereas particles containing no apo E bind to LDL receptors with much lower affinity and remain in the blood much longer. In general, larger particles tend to contain more apo E molecules than smaller ones. This generalization applies to chylomicrons of varying size, as well as those particles that contain apo B-100. However, the affinity of these lipoproteins for lipoprotein receptors such as the LDL receptor is also influenced by other proteins on the lipoprotein surface, particularly the C apoproteins (12) and, presumably, by the lipid composition of the surface, both of which may affect the conformation of the receptor-binding domains of apo B and apo E. Indeed, some VLDL particles are not detectably recognized by lipoprotein receptors, even though they contain several molecules of apo E as well as apo B (13,14).

It is thus evident that heterogeneity of those lipoproteins that contain apo B exists with respect to content of apo E, but that the number of molecules of apo E present is not the sole determinant of their affinity for lipoprotein receptors. Because apo B-100 is an invariant component of VLDL, IDL, and LDL and does not move between lipoprotein particles, the metabolism of these classes of lipoproteins is analyzed in terms of the kinetic behavior of this protein, usually after it is labeled by radioiodination. However, until recently, the variable content of apo E has not been taken into account in such studies, even though this variability has been appreciated for a number of years for VLDL (7,14-18).

We have addressed this issue by developing methods to separate lipoproteins that possess apo B-100 and also contain apo E (B,E particles) and

those that do not (B particles) from isolated lipoproteins or from whole blood plasma (8,19). Labeled lipoprotein fractions or whole plasma containing labeled lipoproteins are passed through anti apo E affinity columns under nonsaturating conditions so that virtually all particles containing apo E are bound. These particles can subsequently be eluted from the columns in 3 M NaSCN; with appropriate precautions, these analytical columns can be reused hundreds of times. In order to maximize analytical precision, we measure content of labeled apo B in the whole plasma or isolated lipoprotein and in the fraction not bound to the immunosorber (B particles). Radioactivity in B,E particles is calculated by difference. By this means, we have shown that VLDL, IDL, and LDL in rabbit and human blood plasma each contain B,E and B particles (8,11, and unpublished data). We have also shown that nascent rabbit VLDL present in liver perfusates or isolated from Golgi-rich fractions of rabbit liver contain both types of particle. Since in rabbits virtually all lipoproteins containing apo B-100 are secreted as VLDL (8,20), heterogeneity with respect to content of apo E does not seem to be solely a reflection of post-secretory events. In the case of human VLDL-IDL fractions, B particles separated by heparin-sepharose chromatography differ from those particles that bind to heparin (including B,E, particles) in having less sphingomyelin, cholesterol, and cholesteryl ester and more phosphatidyl ethanolamine and triglyceride (21). Thus properties of the lipoprotein surface evidently distinguish B and B,E particles and presumably affect the affinity of the particles for apo E.

To determine the influence of apo E on the metabolism of lipoproteins containing apo B-100, we have carried out systematic studies of the metabolism of B and B,E particles in VLDL, IDL, and LDL of normal (New Zealand white) and Watanabe heritable hyperlipidemic (WHHL) rabbits. Because we found in preliminary experiments that separation of the classical lipoprotein classes of rabbits by ultracentrifugation is incomplete, we measured radioiodine in apo B-100 in whole plasma after injection of radioiodinated LDL, IDL, and VLDL. From averaged data obtained from groups of animals, we were able to derive, by use of the SAAM program, a model solution for the metabolism of B,E and B particles in LDL after injection of labeled LDL. We then obtained a solution for the metabolism of these particles in IDL and LDL after injection of labeled IDL, which was consistent with the LDL model. Finally, we obtained a solution for the metabolism of those particles in VLDL, IDL, and LDL after injection of VLDL, which satisfied the combined kinetic data as well as the observed steady state masses of apo B in B,E and B particles in all three lipoprotein classes. Our results in normal rabbits, which have been reported (8), indicate that B,E particles in VLDL are removed more rapidly from the blood than VLDL-B particles; conversely, more B particles are converted to IDL and eventually to LDL. Lipolytic processing of B,E particles is accompanied by partial conversion to B particles, but some IDL and LDL B,E particles are removed from the blood as such. In WHHL rabbits, similar processing occurs, but the rate of direct removal of both B,E and B particles in VLDL is much reduced and greater fractions are converted to IDL and LDL than in normal rabbits. We have been able to fit the kinetic data, obtained in both groups of rabbits, to the same model, in which all particles enter plasma as VLDL and in which some B,E particles are converted to B particles during lipolytic conversion to particles of higher density. The model solutions have permitted us to estimate rates of secretion of B,E and B particles in VLDL and of formation of these particles in IDL and LDL which satisfy both the kinetic and mass constraints of the

system. We have thereby been able to obtain strong evidence that the formation of LDL particles from VLDL is increased approximately threefold in WHHL rabbits, even though the rate of secretion of apo B from the liver is moderately reduced. The model solutions can explain the progressively increasing concentrations of apo B-100 in VLDL (threefold), IDL (fivefold), and LDL (twenty-two-fold) in WHHL homozygotes as compared with those of normal rabbits. A surprising feature of the lipoprotein distribution in WHHL rabbits is that more than one-half of all particles containing apo B are B,E particles, including one-half of LDL particles.

In normal and WHHL rabbits alike, we have been unable to demonstrate a clear effect of the presence of apo E on the rates of irreversible disposal of IDL and LDL from the blood. Mass analyses showed that B,E particles in these lipoprotein fractions contain only a single molecule of apo E. We have therefore concluded that a single molecule of apo E does not materially affect the metabolic fate of IDL and LDL, even though such B,E particles contain two potential binding sites for the LDL receptor. By contrast, we were surprised to find that the irreversible disposal rate of B,E particles in VLDL was greater than that of corresponding B particles in WHHL homozygotes as well as in normal animals. Additional studies in which subfractions of VLDL, differing in mean particle size, were injected showed that rapid removal of VLDL-B,E particles from the blood of WHHL rabbits is largely confined to particles larger than 450 Å in diameter. B,E particles in VLDL of both normal and WHHL rabbits were found to contain on average about three molecules of apo E, but the larger particles contained more. It therefore appears that VLDL may be removed efficiently from the blood of WHHL homozygotes provided that they are sufficiently large and contain several molecules of apo E. In view of the fact that the metabolism of chylomicrons (which also are large and contain several molecules of apo E) has been shown to be normal in WHHL homozygotes (22), we have postulated that the large VLDL-B,E particles may be removed by the chylomicron remnant receptor in the liver (9,11). The mutant LDL receptor in WHHL rabbits, in which four amino acids are deleted from one of the putative receptor-binding domains of the protein, is slowly glycosylated and it is thought that little is transported to the cell surface (23). However, some of the mutant receptors are eventually terminally glycosylated and may be transported to the plasma membrane, even though no receptor-dependent catabolism of LDL has been detected in WHHL homozygotes (24-26). A somewhat analogous mutation of the LDL receptor in humans, in which one of the seven domains thought to contain binding sites is deleted, has been shown to possess abnormal binding properties for lipoproteins: this receptor is able to bind very little LDL, but it binds rabbit beta-VLDL (which contain several molecules of apo E) with virtually normal affinity (27). It therefore is reasonable to postulate that abnormal LDL receptors on the surface of hepatocytes in WHHL rabbits do interact effectively with large VLDL, mediating efficient endocytosis and catabolism of these particles.

The methods that we have devised to study the effect of apo E upon the metabolism of lipoproteins containing apo B do not permit us to determine directly the influence of number of apo E molecules upon receptor-mediated events. However, the results that we have obtained in rabbits indicate that a similar approach to the study of apo B metabolism in humans could yield new insights into the regulation of lipoprotein production, interconversions, and removal from the blood. Given the clear influence of apo E upon

the interaction of lipoproteins containing apo B-100 with the LDL receptor *in vitro*, and the functional consequences of the presence of apo E that we have observed in our experiments with rabbits, this aspect of lipoprotein heterogeneity cannot be ignored in future studies *in vivo*.

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Studies of VLDL Apolipoprotein B Metabolism in Humans: Use of Different Isolation Methods to Characterize VLDL Heterogeneity

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Introduction

Very low density lipoproteins (VLDL) comprise a wide range of particles varying in size, density, lipid and apolipoprotein (apo) composition (1,2). Until recently, studies of the kinetics of apoB metabolism in VLDL have utilized the whole d< 1.006 range of particles (S_f 400-20) as tracer, and although several groups have developed mathematical models that have incorporated some aspects of the complexity within VLDL (3-6), few studies have addressed directly the physical-chemical heterogeneity in this LP class. Because of the diverse nature of VLDL heterogeneity, several approaches to the study of this heterogeneity exist. In this review we will compare several methodologies, focusing on our own work with two of these approaches: separation by flotation rates and by immunoaffinity chromatography.

VLDL Separation by Gel Permeation Chromatography

*Fisher et al (7) used gel permeation chromatography to follow the kinetics of apoB metabolism in different size subpopulations of VLDL and IDL after injection of radiolabeled leucine as a precursor of apoB. This method, which was extremely laborious and made more dificult by the dilution of sample over the columns, allowed the investigators to determine that apoB containing LPs entered the plasma across a wide range of density, including IDL and light LDL.

VLDL Separation by Flotation Rates

The use of gradient density ultracentrifugation protocols, such as that developed by Lindgren and his colleagues (8) allowed investigators to begin to divide VLDL into subpopulations, albeit by arbitrarily defined guidelines, according to their flotation characteristics. Reardon et al (9) used this method to study S_f 400-60 and S_f 60-12 metabolism, noting that all of the former appeared to be converted to the latter in all subjects. However, while S_f 60-12 apoB was converted to LDL efficiently in normal subjects, this conversion was incomplete in hypertriglyceridemic subjects. At the same time, Shepherd and Packard and their coworkers (10) began to carry out similar studies, leading most recently to their detailed investigations of VLDL S_f 400-100, 100-60, and 60-20 apoB kinetics (11). In the latter study, these investigators concluded that little of the VLDL S_f 400-100 was converted to LDL and that most LDL was derived from apoB that entered the plasma on LPs within the S_f 100-20 range.

Because of the significance of their conclusion (11) that VLDL of varying size and density were predestined to quite different fates (indicative of the existance of metabolic channeling), we undertook similar studies in our laboratory. In our initial studies, we observed that apoB specific activity in the three subclasses was not the same. This observation, which stands in contrast to the long held belief that labeling of the VLDL tracer was homogeneous has now been observed in our laboratory in four kinetic studies and numerous in vitro studies. Data from the kinetic studies are depicted below in Table 1.

These results indicate that the mathematical analysis of apoB SA data in VLDL S_f subclasses will be complicated. Furthermore, analyses of studies in which only whole VLDL has been labeled and injected will have to consider this nonhomogeneous labeling in any models of apoB kinetics. We have begun to address this issue in our laboratory and would note that differential labeling of subpopulations of VLDL would offer an explanation for our observation that the area under the apoB SA curve for IDL is frequently larger than that for the apparent precursor, whole VLDL (12). If VLDL were comprised of two or more subpopulations of particles

that had different SAs, and if the proportion of these subclasses that was converted to IDL differed, then the IDL apoB SA could reflect more closely the SA of the subclass that was its major precursor. If that subclass had a higher SA for apoB than the other(s), the apoB SA of IDL would be greater than that expected based on the observed apoB SA in whole VLDL. The latter would be a weighted mean of the apoB SAs of the various subclasses in VLDL. Further studies are in progress in our laboratory aimed at documenting this labeling heterogeneity. In addition, we have begun to develop a compartmental model that can account for such heterogeneity, and that can estimate the range of SAs differences between subpopulations of whole VLDL that would be compatible with the observed SA data in whole VLDL and IDL.

Immunoaffinity Isolation of Apolipoprotein-Specific Subclasses

A completely different approach to the problem of VLDL heterogeneity is the separation of VLDL subpopulations according to their apolipoprotein composition. Specifically, interest would focus on the kinetics of apoB in subclasses of VLDL that varied significantly in their content of apoE, apoCII, and apoCIII. These apolipoprotein-specific subclasses of LPs would be likely to have very different fates, depending on the presence or absence of specific apolipoproteins. Because of the varied, but crucial roles that apoE appears to play in the regulution of plasma LP transport, several groups have attempted to study the metabolism of LPs enriched-in or deficient-in apoE. Nestel et al (13) and Huff et al (14) utilized heparin-Sepharose to separate VLDL into subpopulations relatively enriched and deficient in apoE. Nestel (13) reported that the apoE enriched particles were smaller and cholesteryl ester enriched, and appeared to be the immediate precursor of IDL. These workers also reported that the apoE enriched VLDL seemed to be the product of the metabolism of apoE poor VLDL.

While heparin-Sepharose did differentiate between two populations of VLDL, a more specific approach to the isolation of apolipoprotein-specific LP subclasses would be immunoaffinity chromatography. Havel and his colleagues have presented the results of studies of apoB metabolism in

Table 1

VLDL S_f Subclass ApoB SA (cpm/mg)

Subject	Sample*	VLDL	S_f 400-100	S_f 100-60	S_f 60-20
1.	0	8.4	4.2	6.2	12.0
	3	16.2	5.6	7.9	29.0
2.	0	99.9	149.8	100.6	44.9
	3	19.3	30.5	19.8	10.9
3.	0	17.1	16.8	19.0	9.3
	3	8.9	5.4	9.3	5.1
4.	0	26.4	21.9	21.3	14.1
	3	9.6	7.0	5.8	6.8
5.	3	107.5	152.9	123.7	80.4

*The "0" sample was generated by adding an aliqupt of ^{13}I-whole VLDL to a sample of the subject's plasma obtained just before injection of the remainder of the ^{131}I-whole VLDL into the subject's bloodstream. The "3" sample was a plasma sample obtained 3 minutes after injection of the tracer. The "0" and "3" samples were processed in an identical fashion: VLDL was isolated in a 40.3 rotor and S_f 400-100, 100-60, and 60-20 subclasses were isolated by density gradient ultracentrifugation.

The results indicate that apoB radiolabeling was not homogeneous across the VLDL density range. Both the "spiked" "0" samples and the "3" samples had different apoB SAs in the different S_f subclasses. In addition, the similar relative distribution of the apoB SA in the subclasses in both the "spiked" "0" and in the "3" samples support the belief that this is not an in vitro artifact.

rabbits, isolating VLDL, IDL and LDL that contain and that lack apoE (15). They concluded that VLDL are secreted as two parallel families of LPs, one lacking apoE and one enriched in apoE; that the apoE containing VLDL are catabolized more rapidly and are almost exclusively removed from plasma as VLDL and IDL; that some apoE containing IDL and LDL are converted to their apoE deficient counterparts; and that apoE enriched and deficient LDL are removed at the same rates.

We have been, for some time, attempting to develop methods for the study of apoE-enriched and apoE-deficient VLDL, IDL and LDL in humans. Polyclonal anti-apoE antibodies have been affinity purified by passage over an apoE-Sepharose column, and the apoE-specific IgG has been bound to Sepharose via cyanogen bromide linkage. These columns were first used to demonstrate the existance, in whole serum, of apoE enriched and deficient VLDL, IDL and HDL subclasses (16). More recently, in preparation for in vivo studies of apoB kinetics in apoE-specific LPs, we have carried out validation studies concerned with the isolation and use of these LPs for kinetic studies.

Isolation of an intact particle is a prerequisite for any in vivo turnover studies. In Figure 1, the elution profile of apoE-enriched VLDL from an anti-apoE affinity column with 3M NaSCN is compared to the elution with 0.2M glycine, pH 2.5. It is apparent that NaSCN resulted in the dissociation of apoE from the rest of the particle, while elution with glycine generated an intact particle. We have, therefore, continued to use glycine as our eluting agent.

A second prerequisite for in vivo use of apo-specific LPs is that they retain their native biologic activity. Elution with an acidic agent such as 0.2M glycine might alter such activity, so we carried out an experiment in which apoE-deficient and apoE-enriched VLDL were isolated from ^{125}-whole VLDL, the two separate ^{125}I-apo-specific subclasses were recombined, and the recombined ^{125}I-whole VLDL was injected into a monkey along with ^{131}I-whole VLDL that had not been subjected to affinity chromatography. Figure 2 demonstrates that affinity chromatography did not alter the biologic activity of the VLDL subclasses.

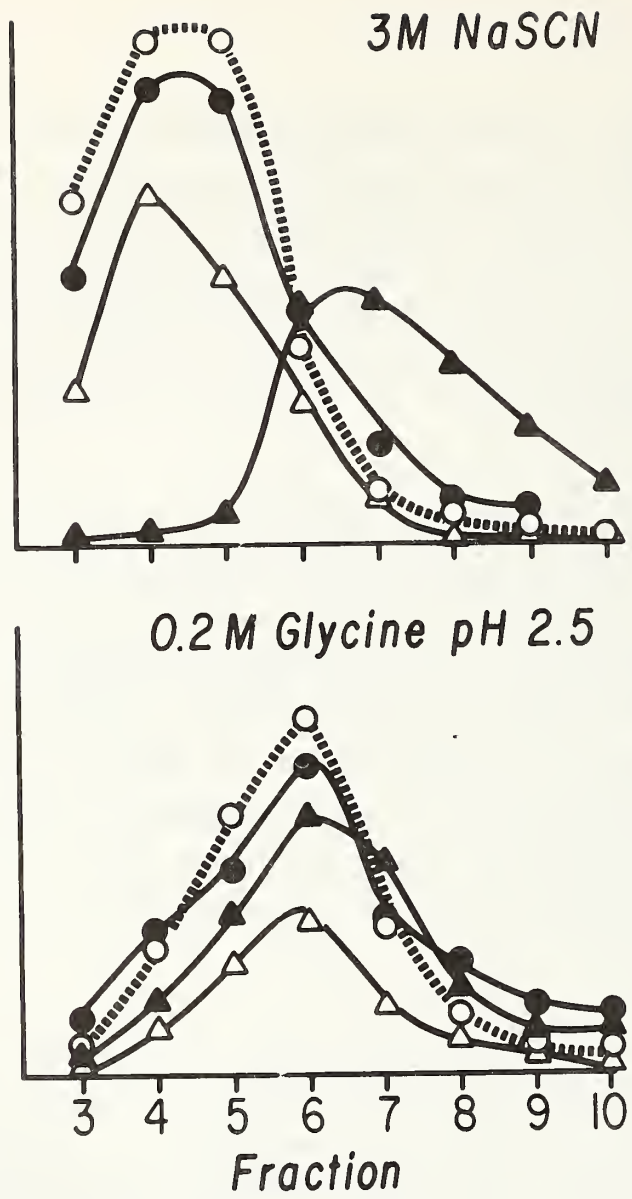

Figure 1. These data depict the elution patterns of apoE-enriched VLDL from a polyclonal anti-apoE affinity column using 3M NaSCN (top Panel) or 0.2M glycine, pH 2.5 as the kaotropic agent. When 3M NaSCN is used, apoE ( ) elutes later than apoB ( ), TG ( ) and cholesterol ( ), indicating dissociation of free apoE from the VLDL particles. When 0.2M glycine is used, all four measured components elute together indicative of the elution of intact VLDL particles. Because of these findings, we have chosen 0.2M glycine as our eluting agent. In addition, the anti-apoE antibodies were affinity-purified by elution from an apoE-sepharose column using 0.2M glycine as the kaotropic agent.

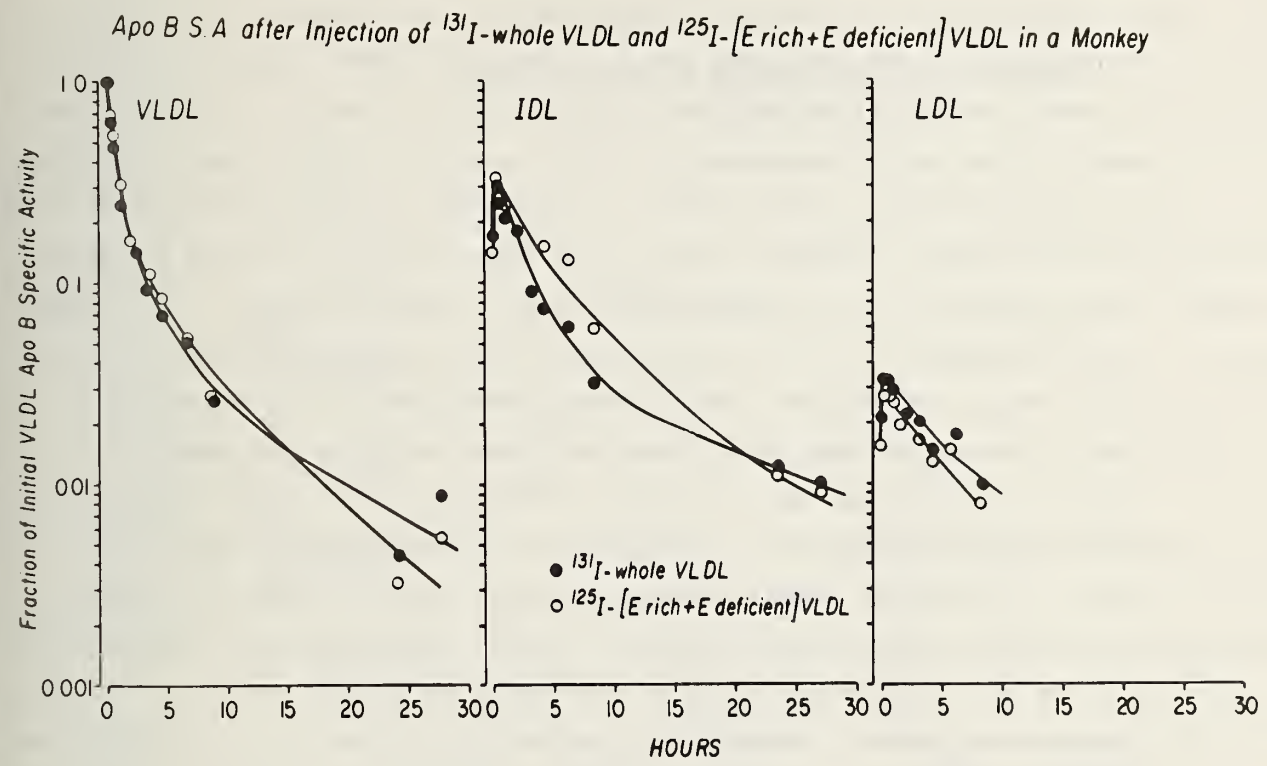

Figure 2. To demonstrate that immunoaffinity isolation of apolipoprotein-specific VLDL subclasses does not disturb their biologic activity, we separated whole VLDL into apoE-enriched and apoE-deficient subclasses by passing it over an anti-apoE affinity column. The apoE-enriched fraction was eluted with 0.2M glycine, pH 2.5. Both VLDL subclasses were dialyzed against saline, recombined, and this recombined whole-VLDL was concentrated in the ultracentrifuge. The recombined VLDL was labeled with I-125 and an aliquot of the original VLDL was labeled with I-131. The plasma curves of apoB specific activity in VLDL, IDL, and LDL depicted above were generated by injection of the two tracers into a monkey. It is clear that the recombined VLDL and the native VLDL were metabolized in a similar fashion.

For these apo-specific tracers to function as markers of the metabolism of particular LP subclasses, they must be stable in vivo. This is critical when one is dealing with exchangeable apolipoproteins such as apoE, apoCII and apoCIII. We believe that although these apolipoproteins can exchange between LPs, this exchange occurs only between LP particles that have the particular apolipoproteins. For example, aopE can exchange between two apoE-enriched VLDL particles, or between an apoE-enriched VLDL and an apoE-containing HDL particle, but apoE cannot move freely from an apoE-enriched VLDL particle to an apoE-deficient VLDL particle. If apoE does leave an apoE-enriched LP, or transfers to an apoE-deficient LP, it does so only after those particles have undergone specific metabolic changes that make it appropriate for them to lose or gain an apoE molecule. To test this thesis, we isolated ^{125}I-apoE-deficient and ^{125}-apoE-enriched VLDL subclasses by affinity chromatography of ^{125}I-whole VLDL and incubated each , separately, with unlabeled whole VLDL. Each mixture of labeled apo-specific VLDL and unlabeled whole VLDL was then passed over the affinity column and apoE-enriched and apoE-deficient VLDL subclasses isolated once again. Figure 3 depicts the results, which demonstrate that in each case, the nonexchangeable apoB radioactivity remained in the appropriate subclass of VLDL.

Finally, if we are to learn anything from these studies, there must be differences between the subclasses isolated by affinity chromatography. Table 2 indicates that there are significant compositional differences between apoE-enriched and apoE-deficient VLDL subpopulations. In addition, these results demonstrate that the isolatin procedure is very reproducible. With these preliminary studies as a basis, we are about to embark on studies of the kinetics of apoB metabolism in apoE-specific subclasses of VLDL, IDL and LDL in our laboratory.

Monoclonal Antibody Characterization of VLDL Subclasses

The use of monoclonal antibodies to characterize subpopulations of LPs according to the presence or absence of physiologically relevant apolipoprotein epitopes on particle surfaces will be the next area of study in many laboratories (17). The combination of such

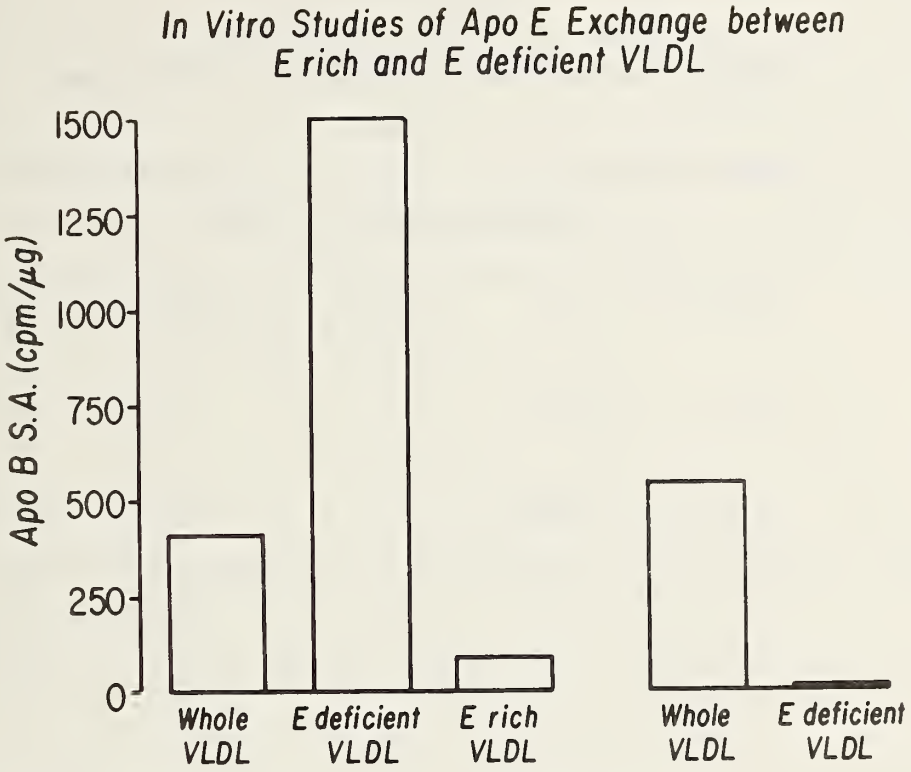

Figure 3. A critical assumption in studies of the kinetics of apolipoprotein-specific subclasses is that they are stable metabolic products. To support this proposal, we isolated radiolabeled apoE-enriched and apoE-deficient VLDL subclasses from ^{125}I-whole VLDL, incubated each subclass separately with an aliquot of the original unlabeled whole-VLDL, and then separated each preparation into apoE-enriched and apoE-deficient VLDL subclasses once again. When ^{125}I-apoE-deficient VLDL was incubated with unlabeled whole VLDL, apoB radioactivity (non-exchangeable) remained in the apoE-deficient subclass (left side of figure). When ^{125}I-apoE-enriched VLDL was incubated with whole unlabeled VLDL, apoB radioactivity did not appear in the apoE-deficient subclass. These studies indicate that each VLDL subclass was stable after reincubation with whole-VLDL.

Table 2.

ApoE-Enriched/Deficient VLDL Subclasses

	ApoE-Enriched (Mean ± S.D., N=5)	ApoE-Deficient (Mean ± S.D., N=5)
% Subclass	32.6 ± 1.7	67.4 ± 2.5
% ApoE	30.4 ± 6.3	7.8 ± 2.1
Chol/TG	0.57 ± 0.13	0.19 ± 0.02
% ApoCIII	37.5 ± 3.5	38.0 ± 3.0
% ApoCII	13.6 ± 1.2	19.0 ± 1.8
ApoCIII/ApoCII	2.9 ± 0.35	1.9 ± 0.21

These data derive from five separate isolations of a single VLDL sample. The apoE-enriched VLDL differ significantly from the apoE-deficient VLDL in their apoE content; their enrichment in cholesterol relative to TG; and their enrichment in apoCIII relative to apoCII.

It is unclear whether the apoE that is present in the apoE-deficient VLDL subclass is the result of contamination by apoE-enriched particles or the presence of masked (unexposed) apoE on another population of VLDL particles. We favor the latter explanation.

characterizations with kinetic measurements of the metabolism of those subpopulations will afford the unique opportunity to attempt to link, directly, kinetics and composition. Use of monoclonal antibody affinity columns to isolate subclasses of VLDL (for example), that have, exposed on their surface, the epitope of apoB involved in binding to the apoB,E receptor, should allow for the labeling and injection of those subclasses for in vivo kinetic studies of their metabolism. Mathematical modeling of such kinetic data may allow, for the first time, the assignment of physiologic probabilities to the arrows directing various LP pools with other LPs or with exits from plasma.

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DR. GRUNDY: Dr. Packard, we tried to do turnover studies in homozygous FH patients. However, since their VLDL is so abnormal in composition, there is probably so much remodeling in the lipoproteins that maybe it's not valid to draw any conclusions from the VLDL kinetics. The particles that you see may have been around a long time and remodeled a lot. What do you think?

DR. PACKARD: If you sub-fractionate the VLDL fraction and look at the large VLDL, they actually have the same residence time as normal. So, that's reasonable and then you can see that the formed remnants are removed very slowly, so at least you can look at the upper part of the spectrum. The lower part of the spectrum, are around a long time, but what you see is a reflection of what's there to a certain extent. So, you may as well do the experiment and then worry about the results afterward.

DR. GINSBERG: Dr. Packard, two questions. One relates to the CHD study. If the metabolism of the largest VLDL subclass is regulated by ApoE predominantly, would you expect to see less of an effect of CHD because of exchange of ApoE with the unlabeled material in the patient? Secondly I am a little confused about the conclusion you drew on the second FH homozygote patient. Those were specific activity data. They did not show crossing of the curves which is consistent of input of unlabeled material other than from your precursor which is a labeled pool. It is not necessarily consistent with no conversion.

DR. PACKARD: In answer to the first question, when we did those modified VLDL turnovers, we tried very hard to do two things. We tried to exchange out all of the E and replace it with good E in vitro before we actually reinjected the material. We found that the ApoE exchanged very slowly, especially when it was modified with cyclohexanedione. It was quite a slow exchange. But I agree. If that initial lipolysis is dependent upon E, then you have to assume that you can't actually tell what's happening. It is only B-related binding events that you're going to pick up to keep the definition fairly true.

DR. GINSBERG: The other was the non-crossing over the curves.

DR. PACKARD: The specific activity curves are only there for consumption because that is what people like to look at when they look at FH homozygotes. When you do the modeling and you look at the delipidation pathway, VLDL through IDL to LDL, then the IDL to LDL conversion, you can do the analysis which accounts for only five percent of LDL synthesis in those group of FH homozygotes that we seem to think are receptor-negative, whereas it is probably more than 50 percent of LDL synthesis in those group of subjects that are typical of the upper panel.

DR. GINSBERG: So, the rest of the LDL in those lower panels--

DR. PACKARD: Is either coming from IDL or LDL S_f 12 to 20 or S_f 0 to 12.

DR. GINSBERG: Have you injected S_f 400 to 100 in those patients and looked at conversion?

DR. PACKARD: Yes. Four 400 to 100 passes through the small VLDL and the conversion is a small amount. It is more than in normal. So maybe there is a little shunting that goes on there, but it is not sufficient, you are only accounting for about ten

percent of the LDL synthesis from all of the VLDLs. So, the gap is huge and cannot be filled by anything other than some direct input from direct IDL or LDL synthesis.

DR. GRUNDY: But you don't know the size of the synthesis of the large VLDL. It could contribute a lot. You don't know the magnitude of the synthetic pathway of the large. So, even if only ten percent is converted, if the magnitude of that pathway is large, it could contribute.

DR. PACKARD: If you argue from the catabolic data to try to figure out a synthetic rate, it doesn't come up to very much.

DR. DECKELBAUM: Dr. Packard, I expect that the VLDL in type II homozygotes would be quite cholesteryl ester rich. In that situation you have a particle that doesn't have any plasma enzyme which is going to break it down. The only way it is going to be removed is either to transfer it to other particles that can be removed or it will sit there and then it will be dependent on the receptor. Also, in the type IV hypertriglyceridemics the cholesteryl ester content is increasing in VLDL. Have you expressed or tried to relate your results to cholesteryl ester content per particle to see if this gives you some insight as to the clearance?

DR. PACKARD: The homozygotes were all uniform on the cholesteryl-rich small VLDL. They were remarkably cholesteryl ester rich and they were all uniform. In all seven subjects, we couldn't relate the cholesteryl ester content of that small VLDL to its subsequent metabolic fate. We observed that you could put them under the negative bucket or the defective bucket. But lipoprotein lipase or whatever lipase is involved would act very slowly on these particles. But a lot of them in several subjects did make it through. Even 50 percent made it through to VLDL. The remarkable thing for us was the time it took to get that conversion going. It hardly seemed possible. When we look at type fours on that pathway, the size of the remnant that is formed from large VLDL, as you have indicated, would be largely due to the amount of cholesteryl ester that gets in that particle which is related to the activity of lipoprotein lipase in that spectrum. In normal individuals, a lot of IDL is probably formed as remnants and then shunted out sideways. The remnant is nearer the S_f-12 boundary of intermediate lipoproteins and then it is removed directly. In hypertriglyceridemic subjects where the VLDL picks up much more cholesteryl ester during its longer residence, then the remnants are probably bigger and the remnants are cleared as VLDL, not as IDL, which is where the difference is that you found in the 1978 work.

DR. KRAUSS: My question relates to the issue of direct synthesis of LDL as S_f 0 to 12 or IDL and, since you quoted from my slide, I couldn't resist mentioning some of our observations in FH homozygotes and heterozygotes which suggests that the common species in plasma is homologous to our IDL2/LDL1 spectrum which is yet a third channel which can't be resolved. It is entirely possible that that represents the smallest particle that can come out and still make LDL and it may be either close to or the same as LDL. The interesting thing is we find this in non-FH individuals as well. It may be one of the equivalents of the so-called beta VLDL type particles.

SPEAKER: Dr. Havel, have you taken LDL from the Watanabe that is enriched in ApoE and injected it into New Zealand white and seen the rate at which it was cleared?

DR. HAVEL: We've done a few of those experiments and there's no difference.

SPEAKER: It's not cleared any faster than the LDL?

DR. HAVEL: No, it's not.

DR. SNIDERMAN: Dr. Havel, could you amplify a bit on the apparent differences between the results in humans and the Watanabe rabbit with regard to direct synthesis because it seems to me they vary importantly on the problem. You can account for everything in the Watanabe, an altered SEI? In the human, there is apparently still direct synthesis and I don't see a technical problem in the human studies to account for the difference.

DR. HAVEL: If I were David Chames, I would say the simple integral solutions of kinetic data in the presence of lipoprotein heterogeneity cannot be relied upon. Now, translating that, if you've got kinetic heterogeneity, you may not get simple precursor relationships. Also, even more important, not being David Chames, I would say that technical problems get in the way and a lot of these may relate to the deficiencies of the centrifugal separations. You get down to five or ten percent of your initial injected ApoB in the plasma, at that point you are looking at products being formed. You don't have to have very much contamination to get completely, as they say in the trade, fluxed up. Pardon the expression. In rabbits we can certainly do experiments of that sort. We did that initially in centrifugation and we got the same results Ghiselli reported. We concluded major input of ApoB is LDL. That doesn't say that that also applies to humans. That remains to be tested, but the approach that we've applied here in the rabbit can be tested in humans and applying what we learn in the rabbits may allow us to address that more directly. We've looked at a lot of the recent literature that's been published regarding ApoB kinetics in many species. This problem is wide-spread. A lot of this issue of independent secretion of ApoB may be confounded by kinetic heterogeneity together with technical problems, separation of laboratory fractions in various combinations, but the only model we study is the rabbit. I'm sure about the rabbit but I'm not sure about any other species.

DR. SCANU: What happened with the Watanabe rabbit with estrogens or during pregnancy? Can you change the situation?

DR. HAVEL: That's been published recently by the Dallas group. They have given large doses of estradiol to normal rabbits and it increases LDL receptors to between five and ten-fold. We confirmed that. We are currently in the middle of kinetic studies in those animals.

DR. SCANU: In Watanabe rabbits, what happens?

DR. HAVEL: Nothing.

SPEAKER: I have a comment on Watanabe rabbits. We have given estrogen to Watanabe rabbits and they have responded. The LDL, IDL, and VLDL has gone down.

DR. HAVEL: Oh, you meant a concentration. They don't eat very well and they even get ascites if you continue the estrogens for more than six days in our experience. They lose weight. The rats in that model also lose weight.

DR INNERARITY: Dr. Havel, you postulated that the large VLDL in the WHHL rabbit may be going through the remnant receptor. Could you tell by your kinetic data whether something is going through a receptor or not?

DR. HAVEL: No. We haven't got any data to show saturability, for example. We're simply going by the rates and what is thought to occur in normal animals. What

happens when you inject the large VLDL into Watanabe rabbits--it's important to emphasize that more than 90 percent of the ApoB is B-100. If you don't do that, obviously you don't know what you're doing in the large VLDL. About 50 percent disappears in a matter of minutes and then it is flat as a pancake from there on out. There is obviously heterogeneity within that class. We don't see that with large VLDL in normals. That is almost monotonic and it is extraordinarily fast. Those results in normals are very akin to what was referred to this morning, the work of Stalenhoef in humans. We find almost exactly the same picture that we saw in the human studies for large VLDL in normals.

DR. INNERARITY: That was beta VLDL instead of HDL.

DR. HAVEL: But it showed that. That's what my slide would have looked like if I had shown it.

DR. FISHER: Dr. Ginsberg, once again I commend you on beginning to sort out some of these parameters that those who label with iodine have to worry about. I am intrigued by your exchange transfer experiment in which you iodinated your purified fractions of VLDL. Have you by any chance added these fractions back to whole plasma to see whether if you incubate in plasma you can carry out these sorts of exchanges of ApoE?

DR. GINSBERG: We haven't done those in vitro studies yet. We do have some other in vivo studies. We usually start off doing the in vivo studies and then go back and do the in vitro studies. In two or three very early in vivo studies that we did, at the earliest time point there was very little specific activity in ApoB in the opposite fraction, much less than you would expect based on the fact that from in vitro studies that we've done exchange of ApoE radioactivity is almost instantaneous. So, whenever E exchanges in plasma, it exchanges extremely rapidly. We drew a sample at five minutes and we saw very different specific activities in E-rich and E-poor VLDL after injection of either one of those subclasses. So, I think that supports the incubations that I've done, but I need to do more.

DR. PACKARD: Can I make three points just on that last point that Dr. Fisher made. If you inject type three and type four VLDL, big VLDL, and follow them through in the one individual, the two show independent kinetics. So, I think there is very, very slow exchange, if there is any at all, between these kind of particles.

Secondly, on your delipidation scheme with the metabolic channeling if there are equal losses all the way along, it will look as if you're not getting the big material through that into LDL. But most of the kinetics show in normal individuals that if you label big VLDL that it is quantitatively converted into the smaller remnants. So then you are comparing like with like if you directly label the smaller remnants. You haven't got any losses initially. The conversion is complete. So, if the large particles go to the 20 to 60 and then you compare remnants from the 20 to 60 with direct labeled 20 to 60 VLDL, they should show the same kinetics and they don't.

DR. GINSBERG: That supports your hypothesis. There is still the possibility, as you've attempted to look at 20 to 60, is 20 to 30, 30 to 40, 40 to 50, et cetera, et cetera. Since this is a continuum, dropout can occur in a continuum. We've injected a huge amount of radioactivity into monkeys as 400 to 100 and attempted to reisolate that label at 20 to 60, reinject it into a second animal with differentially labeled

20 to 60 isolated as such. We've just started that experiment. It is very difficult technically to do, but maybe that is the kind of approach that will give us some idea because if the 20 to 60 is there wherever it came from it ought to do what it is going to do.

DR. PHAIR: Dr. Havel, it seems to me that your model also implied that there was not free exchange of E from one class to another, that if he had injected B only and if there were free exchange, you would have had activity moving over to the other channel. Is that true?

DR. HAVEL: We've modeled the exchange possibility and it doesn't work. If there is rapid exchange, you couldn't get the different differential behavior. We've also done the in vitro incubation experiment which shows that it doesn't occur. I'd like to comment about specific activity. That is a terribly important observation. We have looked at that very specifically in rabbits and at least I can tell you that the B and the B/E particles in each density fraction have the same specific activity. If you don't have that, you don't have a tracer or you have to take that into account.

DR. GINSBERG: We haven't looked at an E-rich and an E-poor VLDL yet.

DR. GRUNDY: Dr. Packard, I think you found something different from that. You found they had all the same specific activity.

DR. PACKARD: That was my third point. We looked at that. We did normals, type fours and type fives and we found differences in the C proteins, but at the B, we found a fairly uniform specific activity.

SPEAKER: Did you both use the same method for measuring the B?

DR. PACKARD: We did TMU precipitation and then measured the specific activity in the B.

DR. GINSBERG: We're trying to compare TMU as a method with gel chromatography and we've just started doing that.

DR. GRUNDY: You shouldn't do any more experiments until you solve that problem.

SPEAKER: Has anybody else looked at it?

DR. NESTEL: I've looked, but only at two fractions, 20 to 60 and 60 to 400 and with isopropanol precipitation and they were identical.

DR. GINSBERG: In addition to these five studies, we've done about ten simple in vitro studies spiking VLDL in varying levels of specific activity and we consistently see differences. Really I hope it is a methodologic problem.

SPEAKER: Have you taken the same sample and run it a couple of times. Because you've got them going different ways, some of that could be analytical variation.

DR. GINSBERG: No, we have reproducibility studies, samples in triplicate, samples run on different days, with the same results.

DR HAVEL: There is some literature on human heterogeneity with respect to E that came out fairly recently from Dr. Phoebe Fielding's laboratory. She uses an approach somewhat similar to you in that she fractionates the plasma first, but in this case

using heparin Sepharose chromatography and spins up the different fractions. She is able to get fractions essentially free of E by the way she applies that technique. She's observed some very distinct differences not only such as you've shown and which the Fielding laboratory has published previously with respect to cholesteryl ester triglyceride ratio; but she finds very striking differences in content of phosphatidyl ethanolamine being three times higher in the B particles than in the E particles and also they have lower sphingomyelin content by about a third.

There is some information about B/E, the physical property of B/E which is much more like cholesterol. This may have to do then with the ability to accept cholesteryl esters and also perhaps to accept ApoE. So, ApoE isn't on there just by chance. It is very real and it relates to particle composition; how it relates is yet to be determined. Also, since we work with rabbits we have certain advantages. One is we can look in liver perfusates, we can look in Golgi apparatus and there are B and B/E particles in single pass liver perfusates from rabbits, mainly B/E. In the VLDL of course, that's what comes out. But there is about a quarter B particles in those perfusates, both from normal and Watanabe animals and, even though they are single pass, we wondered what's really in the liver, what is really on the way out. We find up to about a third B particles in the liver, but two-thirds B/E particles in highly purified Golgi fractions that Bob Hamilton prepares and we are still in the process of characterizing the phospholipid composition of those particles, but the concept is beginning to emerge that those particles are hetergeneous from the time they are synthesized and then the question rises how can that happen?

Well, there is metabolic channeling within the liver cell and there are liver cells that are near the central vein and those that are near the hepatic triad and there is heterogeneity around the hepatic sinosoidal cord, but that's a hypothesis. But the heterogeneity of VLDL goes back into liver itself. I have a question for Dr. Ginsberg. Have you compared heparin sepharose chromatography with your columns directly in the same samples? We're doing that. We see some differences. Do you have any experience on that?

DR. GINSBERG: No, I was never able to to separate VLDL on heparin sepharose. We used to make our own heparin sepharose all the time for lipoprotein lipase purifications and we tried to use that to follow up Paul Nestel's work. For whatever reason, the heparin that we were using or the methodology that we were using, we used to get all the VLDL to bind. We also had LDL binding. I just dropped it at the time and I haven't gone back.

DR. HAVEL: We had to work very hard to get a proper polyclonal antiserum that would see all the E in the population. So, selection of antisera may be also critical to the results you get with this approach.

DR. NESTEL: Dr. Havel has already raised this question of B and E-rich and E-poor particles being channeled independently in the liver and being secreted as such. Peter Alaupovic might like to comment on his Hep-32 cells. In your last slide, you made a suggestion that one might be derived from other. In our initial heparin sepharose state, I also reached that conclusion. More recently, we've done a series of studies in which we've also endogenously labeled the triglyceride as well as the ApoB exogenously. Whereas the ApoB gave the appearance of a possible precursor product relationship between ApoE-deficient and ApoE-rich particles, the triglyceride kinetics clearly show that there was very rapid, newly secreted material into both fractions occurring at the same time with roughly the same FCR. So, we at least found that we had to have a measure of endogenous labeling to be able to determine

this and from that point on we could then model the likelihood of a precursor product relationship, but we found two streams of independent particles down to a large VLDL remnant in which both sets of particles channeled into that common remnant.

DR. RUDEL: Since Dr. Havel mentioned VLDL heterogeneity and since our results in monkey liver perfusions are quite different from his results in rabbit liver perfusions, I thought I should just mention the possibility that a VLDL is not a VLDL which sounds brand new for this conference today since we've been talking about heterogeneity. There are particles in monkey liver perfusates which do not look like VLDL's compositionally, but which do not float at 1006 and whether or not they are VLDL's which are modified during recirculating liver perfusion we checked by doing non-recirculating liver perfusions and they are present in non-recirculating liver perfusions. They are particles with ApoB and E as the two primary lipoproteins. They are triglyceride-rich in terms of neutral lipid, but they do have extra surface. They have extra phospholipid and those particles would not be isolated by centrifugation at 1006. I don't know in how many situations those particles might be present. They are not found in the rabbit. Whether or not they are found in other species other than the monkey, whether or not the liver perfusion reflects the in vivo situation I don't know. But in terms of lipoprotein heterogeneity we have to remind everybody that there are potentially precursor lipoproteins secreted by primate livers that may be candidates for indirect secretion of ApoB into non-VLDL fractions.

SESSION V. Metabolism *In Vivo*, Continued

Chair: Waldo Fisher

In 1972, and more formally 3 years later, Phair and Berman presented the first kinetic model of apoB which showed heterogeneity in the metabolism of this apolipoprotein, as manifest by multiple secretory pathways entering polydisperse LDL (Fed Proc 1975; 34:2263).

A few years later, the concept of metabolic heterogeneity in the metabolism of apolipoproteins was dignified by reaching back into the 1950s and resurrecting the phrase "metabolic channeling," originally coined by David Drabkin and Julian Marsh (J Biol Chem 1955; 212: 623-631). The phrase conveys the concept that a chemical substance may be metabolized along alternate metabolic pathways which do not necessarily intersect. Data is mounting to suggest that such may be the case for apolipoproteins. Perhaps, depending upon the composition of the particles on which apolipoproteins travel, their intravascular processing and eventual fate may differ.

Dr. Loren Zech will lead off this morning's session with a discussion of Metabolic Channeling of apoB.

METABOLIC CHANNELING OF ApoB CONTAINING LIPOPROTEINS

or

Analogic Realization of the ApoB Dynamical System

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The concepts discussed in the next few paragraphs are those necessary to understand metabolic channeling. Furthermore, using these concepts, models of apoB metabolism which are under development will be examined. While the rate constants determined, using these models, will not be examined in detail, these models will provide a framework for understanding the application of the concepts of metabolic channeling to apoB metabolism.

What is metabolic channeling? This term was first used by Drabkin and Marsh[1] in the context of a biological system. The apolipoprotein system as we understand it today consists of a system made up of lipids and proteins with particular states, kinetics, and dynamics. The what, why, when, where, and how of metabolic channeling will be understood in these terms. Therefore, the reader and the author need to agree on the concepts represented by the terms state, kinetics, and dynamics. In addition, some framework for an understanding of the interactions between states, the kinetics and the dynamics of moving from state A to state B will be necessary for a complete description of metabolic channeling. These concepts are further understood and described in the context of compartmental models.

Analogical realization: A compartmental model of a particular biological system is at the same time an analogy of the biological system and a summary of the experimental data collected from experimentation on the biological system. Repeating an experiment on a model should yield exactly the same data (within some window of random variation) as was used in constructing the model. From this description of a model come two theorems in the theory of mathematical model building[2].

Theorem 1: There always exists a model.

Corollary 1: There always exists a minimal model.

Theorem 2: Properties of the model are always either
(a) inherent in the experimental data used to construct the model or
(b) completely arbitrary.

The second possibility is ruled out if the model is minimal.

Theorems one and two above interact with the definition of a model in an iterative order such that the minimal model represents a summary of the experimental data. When new experimental data is combined with the model a new minimal model results leading to a better realization of the biological system under examination. There is no disagreement that compartmental models for lipid, lipoprotein, and apolipoprotein metabolism have moved through many such iterations[3].

Among all the techniques of reasoning, analogical reasoning has a privileged position in that it is the essential factor in hypothesis formation[4]. While logicians rightly conclude that analogical reasoning does not allow a unique description of a biological system, as is the case with deductive reasoning, analogic reasoning is a source of knowledge in the context of theorems one and two above. An analogy, and hence a model, may be more or less correct depending on the nature of the experimental data[5]. Furthermore, theorems one and two imply that no false analogies can exist because the model always comes from the experimental data.

Investigations of knowledge-based systems, which are only now being understood through the work of artificial intelligence in conjunction with movement towards a general theory of model building[6], have provided an environment for the development, accumulation, and specification of knowledge about metabolic systems in the analogical sense[4]. These concepts are divided into the two dimensions of knowledge base and inference engine through the application of artificial intelligence techniques as illustrated in fig. 1.

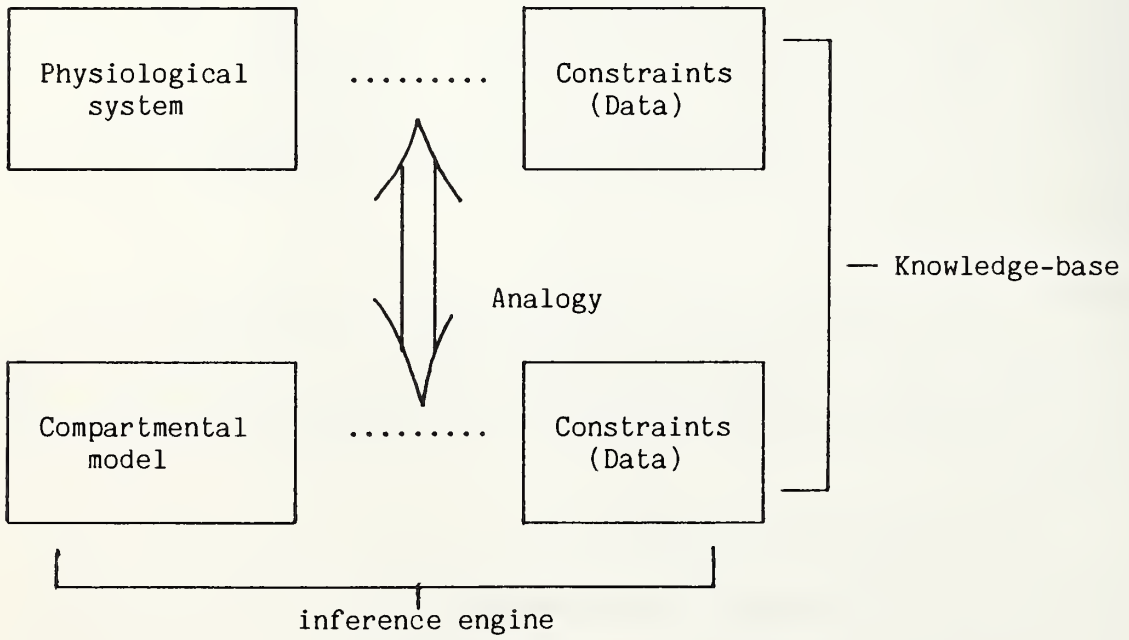

Figure 1) Two dimensional view of modeling.

The realization of the physiological systems, through the application of an analogy based in the general mathematical theory of compartmental models[6], on one hand, and constrained to the real[7] world by the experimental data, on the other hand, will be examined in more detail for the apoB case.

Tracer-tracee relationship: The tracer-tracee relationship is important, as most investigations of metabolic channeling of the lipoprotein system are investigated with the aid of tracers. A most important characteristic of this relationship is that, in the steady state, the tracer follows linear kinetics even if the tracee follows non-linear kinetics[8]. This relationship is best understood by an example. Consider the frequently used example[9] of a second order process or reaction step as given by the equation below:

$$df/dt = -kf^2 \qquad (1)$$

where f is the tracee subsystem and k is the second order rate constant. This is not a first order process. When a tracer is introduced into the system it becomes combined with the tracee and:

$$f = f^* + f_c \qquad (2)$$

where f^* represents the tracer and f_c represents the tracee. Note that for f^* to represent the tracer

$$f^* \ll f_c \qquad (3a)$$

and

$$f \simeq f_c. \qquad (3b)$$

Rewriting equation 1,

$$d(f^* + f_c)/dt = -k(f^* + f_c)^2$$

realizing that,

$$1 = f^*/(f^*+f_c) + f_c/(f^*+f_c)$$

and applying the distributive property of derivatives,

$$df^*/dt + df_c/dt = -kf^*(f^*+f_c) - kf_c(f^*+f_c).$$

Using 3a,

$$df^*/dt + df_c/dt = -kf^*f_c - kf_c^2;$$

from the starting tracee relationship in (1),

$$df_c/dt = -kf_c^2$$

therefore,

$$df^*/dt = -kf_c f^* = -Lf^*. \qquad (4)$$

There are several important concepts in this simple example. First, the expression $L=kf_c$, is only constant if f_c is not changing. If f_c is changing as a function of time, then L is time dependent. Second, even if L is time dependent, the tracer kinetics are first order with respect to f^*. Third, there exists a trade-off between detecting unobservable parts of the system in the process of estimating the rate constants associated with equation 4 and understanding the tracee relationships by estimating the rate constants associated with equation 1. It is important that both the rate constants for the tracee and the topological structure of the model can not be determined from experimental data unless the study is carried out in more than one state of the biological system under investigation.

States: States are usually a measure of the amount of molecules or macromolecular complexes having similar physical properties and location. It is within this definition that a large part of the heterogeneity concepts and arguments take place. Macromocules can be constant with respect to one physical property or location, while differing in another. For example, VLDL particles are in the same density range while they have more or less triglycerides and more or less cholesterol ester. There can even be an exchange of one lipid for the other without movement of the lipoprotein out of the VLDL density range. Overlapping boundaries of state definitions makes the task of understanding heterogeneity difficult.

Kinetics: Even more difficult to understand are the concepts of transport of material (lipid or protein) from one state to another. These kinetic concepts are based on specific understanding of the states on two planes. First, the measure of the state must be constant. This usually extrapolates to having used a constant separation technique for a particular study. This makes study to study comparisons difficult. Second, is the amount or level of lipoprotein, lipid, or apoprotein constant or is it changing while the action of moving between states is underway? In a specific sense, is a lipoprotein particle entering a specific state for every lipoprotein particle moving out of that state? If the answer is yes, the level of state is unchanging, and this situation is designated steady state. If the level of the state is changing, then one is no longer dealing with kinetics of flow into or out of a particular state but rather with the dynamics of the state. In other words, how does the level of state changes from one observation to the next. These concepts have important consequences in the study of metabolic channeling.

Dynamics: Dynamics is the most difficult of the three descriptors of metabolic channeling. This fact results because dynamics describes the changes in state which are a consequence of a change in kinetics. This descriptor is further compromised because of the counter-intuitive processes of feedback and delay resulting in periodic, aperiodic, and catastrophic systems behavior. In addition, the dynamical behavior of a system not only depends on metabolic process but also on the processing and archiving of information about the processing. Two major consequences are: First, the system under study can only

be examined in terms of its forward and reverse control loops when studied under the influence of a clamp, and second, the relationship between a controlling and a controlled state is dependent on the past history of both.

The concept of state formation as a consequence of kinetic and dynamic systems behavior is applicable to biological systems with a few states as well as to systems with many possible states. Because of the large number of states, and because of the small differences between many of the states, combining several states into a larger average state is useful in the investigation of β-containing lipoproteins. For example, VLDL is an average state with respect to triglycerides, cholesterol esters, ect.

To define a particular average state, it is usually best to discuss the average state in terms of the slowest changing physical property of that state. ApoB fills this reference role for the apoB-containing lipoproteins. It must be pointed out that this does not preclude, for example, the changing of LDL apoB levels, rather: 1) apoB levels change in a prescribed manner and 2) that apoB is the slowest changing component of the average LDL state.

Now that the preliminaries are established we consider metabolic channeling of apoB-containing lipoproteins by questioning in some detail the kinetics and dynamics of the Chylomicron, VLDL, and LDL average states.

Chylomicrons: Some controversy exists about the definition of this average state. For purposes of this paper we will define this state as triglyceride-rich lipoproteins containing apoB-48, secreted from the intestinal cell into the lymphatic circulation. A second average state, designated the chylomicron remnant, results. This remnant is a relatively triglyceride-poor, apoB-48 containing, lipoprotein. The chylomicron remnant is a result of triglyceride and phospholipid removal from a nascent chylomicron. Dr. Trevor Redgrave[10] has carried out experiments resulting in the experimental data shown in figure 2. This series of decay curves resulted from the injection of chylomicrons labeled in the triglyceride and cholesterol ester moieties.

The rat is an animal which does not exchange chylomicron cholesterol ester with other triglyceride-rich lipoprotein particles in the plasma. The constraint is made in the model (figure 3) that the cholesterol ester kinetics represent the chylomicron particle kinetics and serve as the reference for the chylomicron state. Cholesterol-ester also represents the chylomicron apoB kinetics if apoB is constrained to remain with the chylomicron while it resides in the plasma. Nascent chylomicrons enter the plasma where triglyceride is removed more rapidly than cholesterol ester resulting in a steep decay of triglyceride compared to cholesterol ester/apoB decay (figure 2). This loss of triglyceride results in a triglyceride poor remnant from which the triglyceride and cholesterol ester/apoB decay more slowly together.

A portion of the lipoproteins are taken up as intact particles, with cholesterol ester and apoB intact, and a fraction of these are taken up by the liver. The liver also takes up a portion of the delipidation products. Not shown in this kinetic model are the phospholipids; these have been shown to be removed to another lipoprotein state, namely HDL[11].

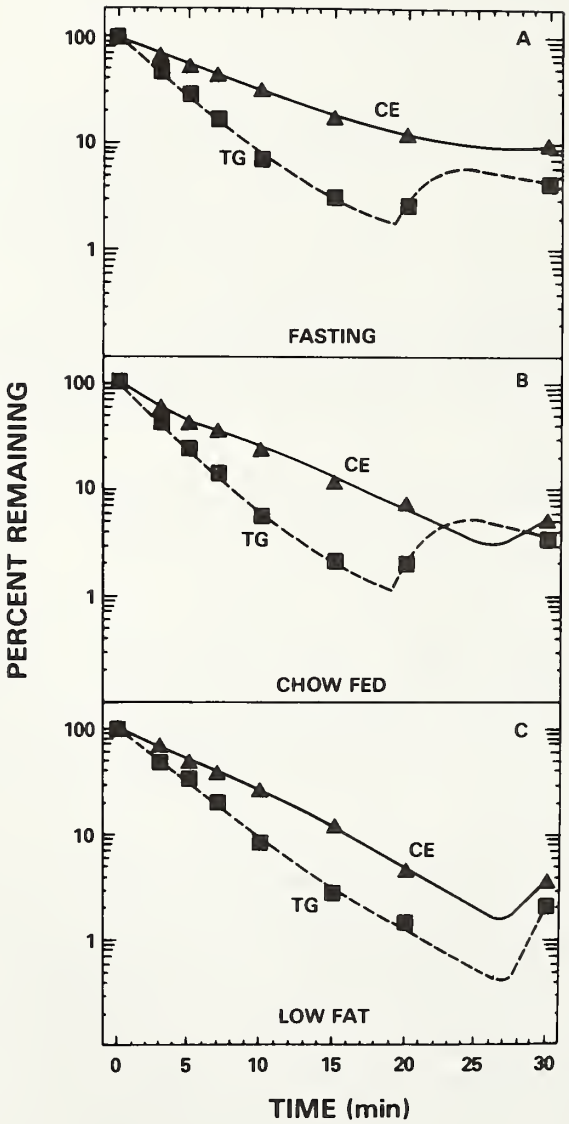

Fig.2- Three examples of rat chylomicron tracer decay curves labeled in the triglyceride moiety ■ and the cholesterol ester moiety ▲.

What are the dynamics of this chylomicron state? The makeup of the labeled chylomicron is narrow with respect to the spectrum of intermediate states formed during metabolism, is it narrow with respect to protein makeup? In the dynamical situation, inputs to the chylomicron state may exist along the delipidation chain (figure 3). There is no reason to believe that the uptake of chylomicrons in proportion to the uptake of chylomicron remnants does not change in a dynamic sense. One of the intentions in building this model was to examine the effect of fatty acid makeup of the chylomicron triglyceride on chylomicron metabolism. This model has had considerable utility in this mode of operation. When extrapolating to the dynamics of chylomicron metabolism it is important to understand that chylomicron metabolism is not a linear process.

If this model is used to extrapolate to the dynamics of periodic feeding, the input does not remain at the top of the delipidation chain but moves to less triglyceride-rich lipoproteins as fat absorption is completed[12]. While the spectrum of apoB-containing lipoproteins may be narrow over any short interval of time, the dynamic range is probably large. What are the dynamics of removal of apoB-containing chylomicrons and chylomicron remnants? A portion of the apoB is removed quickly; the remainder is removed after variable amounts of delay. The removed apoB can be channeled into three possible pathways. The most likely is that a portion is broken down, the least likely is that a portion is reteroindocytosed, and an intermediate possibility is that the chylomicron is sequestered at a receptor until lipoprotein modification has taken place. Depending on which of the mechanisms are chosen, a spectrum of particles are returned to the circulation, probably but not necessarily in the VLDL state.

In summary, the average state of chylomicron apoB-containing lipoproteins has a rapid but distributed kinetics, with a large dynamic range of input and relatively unknown dynamics of removal. In humans, both the kinetics and the dynamics may be more complicated because of the influence of cholesterol ester and of triglyceride exchanges. In the human experience, the apoB continues to be the reference. However, the state of chylomicrons is rapidly and almost completely influenced by the dynamics of lipid metabolism.

VLDL: VLDL apoB lipoprotein metabolic channeling has been examined in many kinetic studies, sometimes by itself but usually in conjunction with LDL turnovers using both exogenous and endogenous labeling. Recently, Dr's Grundy and Fisher[13] studied three subjects in which the kinetics of endogenously and exogenously labeled VLDL were determined simultaneously. Tritiated leucine was the precursor for endogenous labeling and VLDL was labeled exogenously by iodination. Dr. Beltz presented a preliminary model to explain the observations from this study at the AHA scientific sessions in november of 1985[13]. A major finding of this study is the fact that a smaller fraction of the VLDL apoB leucine label arrived in the LDL and IDL when compared to the the fraction of iodinated apoB converted to IDL and LDL. This problem was overcome in the model by having a major portion of the nascent VLDL enter into the end of the VLDL delipidation chain and loss occur at more than one fraction of VLDL metabolism. When VLDL is isolated and iodinated the label is distributed in proportion to the mass of apoB. Because of this distributed labeling, a large portion of the iodinated VLDL is not lost before becoming IDL. In combination, this technique allows the identification of another pathway.

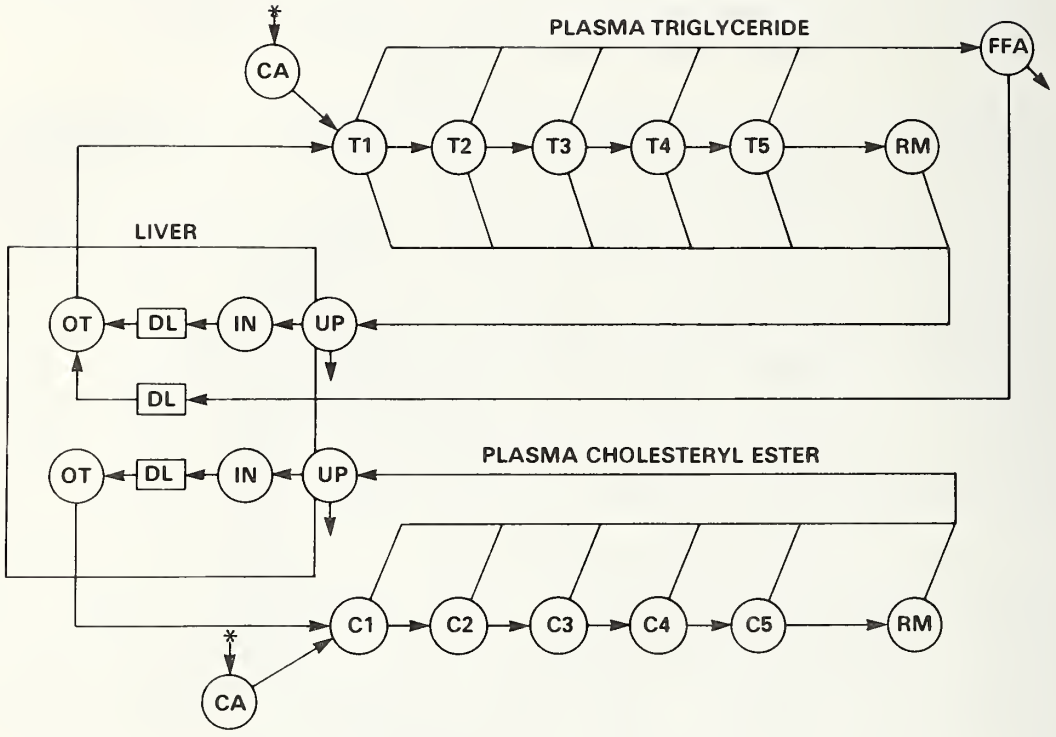

Fig.3- Compartmental model proposed for chylomicron metabolism by Redgrave and Zech[17]. CA=catheter, Tx=triglyceride, Cx=cholesterol ester, RM=remnant, FFA=plasma free fatty acids.

In consideration of the kinetics of the VLDL state, all the differences and problems of the chylomicron state remain. Compared to the spectrum of VLDL isolated from the plasma, is the spectrum of secreted particles narrow or broad? Second, is there more than one non-overlapping spectrum of VLDL lipoproteins secreted? Examining the VLDL kinetics using the leucine tracer has indicated smaller and/or more dense lipoproteins are secreted into the VLDL spectrum of lipoproteins[14]. The kinetics of loss are complicated in that apoB is continuously and irreversibly lost as a slowly-turning-over-species, as well as being lost to the IDL and LDL[9]. The function of the loss pathways of VLDL can only become more complicated as the apoE modulation of VLDL metabolic channeling and the influence of lipid exchange processes on metabolic channeling are included in the kinetics and dynamics.

When VLDL is isolated and iodinated, the label is distributed in proportion to the mass of apoB present. In the process of labeling apoB, all apoB molecules have a probability of being labeled. Is this probability equal or is it modified by the lipids associated with apoB? Is this a source of possible heterogeneity? If the lipids do change the probability, it is likely that the triglyceride-rich particles receive less label, resulting in a change in the shape of the curve for IDL and LDL. This however does not influence the amount of label that reaches IDL and LDL unless there is another loss path which does not provide input for IDL or LDL.

The dynamics of moving from one level of VLDL to another is influenced by changes in the VLDL particle secreted. It has been observed many times that over the course of a few days VLDL particle density decreases, then increases, following carbohydrate induction, cholestyramine treatment, or hormone therapy. The dynamics of VLDL are further influenced on the catabolic side of the coin.

LDL: Because of its importance in the atherogenic process, the quantity and ease of separation from the plasma, and the detailed understanding of the cell receptor, the LDL state has been examined in considerable detail by hundreds of investigators. It is rapidly becoming clear that LDL is made up of more than one state, and each of these states are becoming better characterized by physical-chemical or molecular-biological techniques. Many of these studies are presented in this volume. It is a popular concept that, compared to the chylomicron, the LDL state is well characterized. However using the kinetic and dynamic descriptors of LDL, this state is not well characterized in relation to the real physiological system. For example, are there precursor-product relationships between the LDL subfractions, or are there tight or loose couplings between the LDL apoB and lipid fractions?

The kinetics of LDL subfractions has been under investigation for more than a decade. In collaboration with Dr's Sniderman,Teng,and Thompson I have been developing new models for LDL kinetics. Many other investigators are also presenting new models for LDL kinetics. However, many of the important kinetic concepts were presented in a model first presented by Dr. Waldo Fisher (figure 4). In these studies radioactive leucine was injected and LDL was

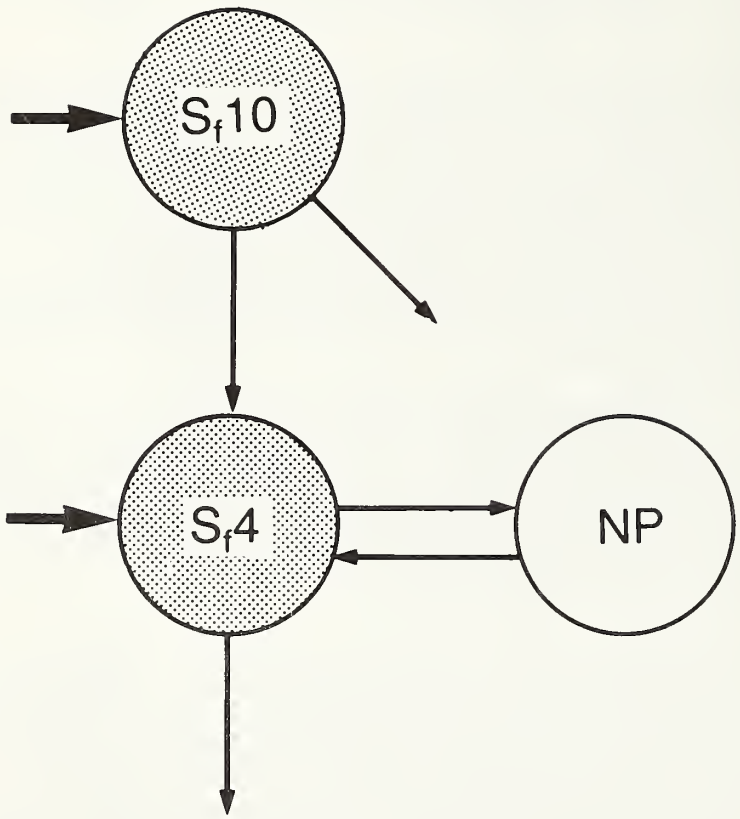

Fig.4- Early model proposed by Waldo Fisher for the metabolism of LDL subfractions.

separated into the S_f-10 and S_f-4 fractions. The kinetics include precursor product relationships between S_f-10 and S_f-4, direct input into both S_f-10 and S_f-4, direct loss from S_f-10 and S_f-4, and an exchange compartment with S_f-4. Using these techniques an exchange compartment with S_f-10 and a precursor product relationship between S_f-4 and S_f-10 were not supported.

Investigation into the dynamics of metabolic channeling of LDL has changed very little over the last several decades. Initial studies examined the state, of LDL before and after treatment or other perturbation, for example, heparin injection. With the development of kinetic models, the kinetics of LDL metabolic channeling were examined before and after treatment or perturbation. A few investigators have examined the LDL apoB level following a perturbent, for example a fat load. Still others have implied the average dynamics by the application of a Michaelis-Menton or saturation type model to population data. Other studies have examined the linear relationship between kinetic parameters such as pool size and synthesis rate. Because of the tracer-tracee relationship, none of the above experimental designs is sufficient to investigate the dynamics of LDL metabolic channeling. The dynamics of metabolic channeling are without a doubt nonlinear. However, even if LDL is shown to have a region of linear dynamical behavior, experimental procedures capable of detecting nonlinear dynamics must be undertaken in order to substantiate linear dynamics. In the process of moving from state A of LDL metabolic channeling to state B of metabolic channeling and back to state A, the kinetics at multiple intermediate steady states needs to be examined. A minimum of one state between A and B needs to be evaluated as the LDL system moves from state A to state B and also as the system moves from B to A. If the system were linear, the intermediate state would be chosen to be half-way between A and B. However, given the probable nonlinear dynamics, the appropriate position and number of states necessary between A and B to determine the system can not be made. When the nonlinearities of the dynamics have been identified post-priori sampling techniques can be delineated.

Because of the heterogeneity of LDL which has been identified up to this point in time, it can be said that the dynamics of LDL are more complicated and will be understood as a combination of dynamics of homogeneous LDL fractions. Simple dynamic studies by Fisher indicated that, after plasma triglyceride was decreased the number of S_f-10 lipoproteins decreased and the number of S_f-4 lipoproteins increased[15]. Because these investigators did not investigate any points between high and low triglycerides, they could not detect the order of change (ie. whether the number of S_f-10 lipoproteins decreased before the number of S_f-4 lipoproteins increases). Dynamical questions such as: Does the increase in S_f-4 lipoprotein number result from an increased turnover of S_f-10 lipoproteins or does the increase in S_f-4 lipoproteins result from a decrease in S_f-10 synthesis and an increase in S_f-4 synthesis. The dynamics of responding to shorter perturbations such as a meal or a heparin injection are even more complicated[16]. As can be appreciated from as simple a model as in figure 4, the systematic study of LDL dynamics will be difficult.

Summary: Metabolic channeling of apoB containing lipoproteins is a complex subject which must be described in terms of, states, kinetics, and dynamics. The average states of apoB, such as chylomicron-apoB, VLDL-apoB, and LDL-apoB, are rapidly being separated into smaller average states by a variety of

separation techniques. In addition, a number of new substates are being hypothesized to explain observed kinetic behavior. The kinetic description of apoB metabolic channeling has only just begun to make a significant contribution to the understanding of this subject even though these studies have been undertaken over a period of several decades. The difficulty of testing kinetic models using experimental data has made the acceptance of the more complex kinetic models slow. Several descriptions and models for the dynamics of apoB metabolic channeling exist, however very little testing of these descriptors using experimental data has taken place. This is almost certainly because of the lack of reasonable tools to study dynamics. While the tracer-tracee relationship results in the application of linear systems theory to kinetic models, even this powerful tool pales in the investigation of dynamical systems behavior. From the point of view of the clinician, it may not be necessary to understand the kinetics or dynamics of apoB metabolism to treat the consequences of lipid and lipoprotein metabolism. It is however almost impossible to have judged as best any treatment or group of treatments without first understanding the dynamics and kinetics of apoB channeling.

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HETEROGENEITY IN LOW DENSITY LIPOPROTEIN METABOLISM

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Introduction

The atherogenic process is complex and multifactoral. Among the numerous recognized risk factors, elevation of low density lipoprotein (LDL) cholesterol is consistently found to be a positive risk factor in almost all population studies. However, many if not most subjects who develop premature coronary artery disease (CAD) fail to have overt elevations of LDL cholesterol. Although it may be argued that most individuals in the western world have LDL levels that are too high, nevertheless there is a great disparity in the incidence of atherosclerosis among individuals who have apparently equivalent levels of LDL cholesterol and other lipoprotein lipid levels. This is true for individuals with high LDL cholesterol, and even among subjects with familial hypercholesterolemia (FH) there is much diversity in the extent of atherosclerosis present and in the rate of progression of the disease.

Since the cholesterol that ends up in the foam cell and the plaque is believed to be derived chiefly from the circulating lipoproteins -- mainly LDL, we, and others, have suggested that there may be important qualitative differences in LDL structure, composition and metabolism that account in part for the enhanced accumulation of arterial cholesterol that occurs in some individuals. In an effort to determine if this idea has merit, a number of laboratories are currently attempting to define, to quantify and to establish the clinical relevance of heterogeneity in the metabolism of subpopulations of LDL, and to determine if any pattern of heterogeneity distinguishes patients with CAD.

We hasten to add the caveat that we do not mean to imply that heterogeneity in LDL will fully account for differences found in the extent of atherosclerosis among individuals with equivalent plasma LDL levels. Clearly, a large number of factors are implicated in the causation of atherosclerosis and in any individual any single factor or combination may be involved. However, we do seek to test the hypothesis that differences in the metabolism of subfractions of LDL may be important in atherogenesis. For example, it could be argued that large LDL particles that contain more cholesterol per particle are more atherogenic than smaller, cholesterol-depleted LDL particles, since the deposition of a fixed number of LDL particles in the extracellular matrix, or uptake into cells of the arterial wall, would result in more cholesterol deposition if the LDL were more cholesterol enriched. On the other hand a greater number of small, cholesterol-poor LDL particles, might be equally atherogenic. Obviously, alterations in production or clearance of LDL subfractions could be responsible for changes in steady state levels of specific LDL particles.

There is now a great deal of evidence for heterogeneity in LDL. Before further discussion it is essential to define exactly what is meant by heterogeneity, as the word has come to be used rather loosely in descriptions of LDL. For many years investigators considered LDL to be a rather homogeneous group of particles found at a given density, i.e., d = 1.019-1.063 g/ml, in which the physical and chemical properties of all the particles were comparable and, most importantly, in which the metabolic properties of all the particles were equal. Furthermore it was widely assumed that all of these properties remained rather constant from one individual to another, as well as constant over time in the same individual. Nowhere has this assumption been more evident than in kinetic studies, in which the metabolic behavior of an autologous LDL tracer has usually been assumed to be invariant. Thus, kinetic studies have been routinely done in which autologous LDL is isolated, labeled and reinjected into a subject in a baseline situation and the parameters of LDL flux calculated. A perturbation such as a drug or a diet is then introduced and after a new "steady state" has been achieved, autologous LDL is again isolated, iodinated and reinjected. New parameters of LDL flux are calculated and any apparent differences in LDL kinetics observed between the two studies are assumed to be due to metabolic changes in the person without any consideration of the possibility that the perturbation changed the intrinsic metabolic property of the LDL itself. As we (1, 2) and others (3-5) have shown, these assumptions regarding homogeneity are probably not valid in many situations. The point is that the physical properties of LDL are not constant from one person to another. Even in the same person, the physical and the metabolic properties of LDL are not constant for LDL isolated at different times under different dietary or pharmacologically induced interventions. In addition there is compositional and metabolic heterogeneity even among the subpopulations of LDL isolated from a single sample (2, 6-11).

For the purpose of our discussion we can define heterogeneity at three different levels: First, we can speak of heterogeneity in LDL found between individuals (e.g., heterogeneity due to polymorphism of apo B or to differences in composition or metabolism). Second, we can speak of heterogeneity in LDL isolated from the same person at different points in time, under different environmental or pharmacologically induced interventions. This heterogeneity could be due to alterations in the proportions of particles normally present, or it could be due to alterations of all LDL particles, or to alterations produced in only a selected subpopulation. Third, heterogeneity may be present in the LDL found in a single sample. Thus, some particles are bigger and more buoyant, some contain more cholesterol or triglyceride and some may contain apo E while others do not. In this paper we will focus on several examples of heterogeneity in LDL at each of the three levels discussed above.

Heterogeneity in LDL

Low density lipoproteins are biological microemulsions made up of apoprotein B, phospholipids, cholesteryl esters and a small amount of free cholesterol and triglyceride. The polar constituents (phospholipids and apoproteins) provide surface stability to a nonpolar, neutral lipid core phase composed predominantly of cholesteryl esters and triglycerides. Traditionally, LDL are considered to be those particles having an S_f flotation rate of 0-12, and currently are operationally defined as those particles isolated at a salt density between 1.019 and 1.063 g/ml (12). This density interval is used because it initially appeared to isolate a class of lipoprotein particles that were rather homogeneous in their physical makeup, at least compared to

particles found at the lower densities, which were markedly polydisperse. However it is clear that within the LDL density range are found particles that differ from each other in terms of size, buoyancy, lipid content, apoprotein content and most importantly, metabolic properties (1-16).

Differences in LDL between individuals. It is now recognized that LDL isolated from different individuals may differ in a number of parameters. On the one hand the apoprotein content of LDL, chiefly apo B, can differ. For example, we have recently shown that an expressed polymorphism in apo B could be detected by a specific monoclonal antibody, MB19 (17). This common polymorphism is accounted for by the codominant expression of two apo B alleles, one allele encoding for an apo B containing an epitope that binds MB19 with high affinity ($MB19_1$) and one allele encoding for an apo B that binds MB19 with relatively low affinity ($MB19_2$). Thus, while homozygotes contain LDL that is homogeneous with respect to MB_{19} binding, i.e., all LDL particles contain apo B from the $B19_1$ (or $MB19_2$) allele, heterozygotes have half of their LDL particles of $MB19_1$ and half of $MB19_2$. The region of apo B that binds antibody MB19 is not involved in binding to the LDL receptor, and the MB19 polymorphism does not appear to have any clinical significance (18). However, in an analagous manner it may be predicted that heterogeneity in the LDL receptor binding domain of apo B would have important clinical consequences, reflected in variations in plasma LDL levels. We have attempted to find polymorphisms in the LDL receptor binding domain of apo B using monoclonal antibody, MB47, an antibody which binds to, or near, the LDL-receptor binding domain of apo B (19), but to date have not been successful.

The composition of LDL is also known to vary among individuals and even between sexes. For example, subjects with familial hypercholesterolemia are usually found to have LDL that are larger in size and cholesterol-enriched (14). In contrast, subjects with hypertriglyceridemia are invariably found to have LDL that are more dense, cholesteryl ester-depleted and relatively enriched in triglyceride (3). In contrast to larger LDL, these LDL have a decreased uptake in cultured fibroblasts, and deliver less cholesterol per particle to the cell. (4). Of great relevance, Sniderman and colleagues (20, 21) have defined a population of patients with a greatly increased incidence of CAD whose LDL are characterized by similar properties. These individuals have normal LDL cholesterol levels, but an increased apo B level, indicative of an increased number of LDL particles. This condition has been termed hyperapobetalipoproteinemia. These subjects, who have an increased number of small, more dense LDL particles, may or may not have accompanying hypertriglyceridemia. Because the LDL are relatively depleted of cholesterol, they have "normal" levels of LDL-cholesterol, though their LDL apo B levels are increased. A preliminary analysis of a survey of CAD patients being conducted in La Jolla also indicates a markedly increased incidence of hyperapobetalipoproteinemia in patients with CAD and in most of these, hypertriglyceridemia does not appear to be present (Young, Beltz and Witztum, unpublished results).

As noted above, patients with hypertriglyceridemia usually have small, dense LDL. Many of these patients also have increased numbers of LDL particles (i.e., increased apo B). Brunzell and colleagues have also reported that subjects with Familial Combined Hyperlipidemia (FCHL) also have increased plasma apo B levels, irrespective of lipoprotein phenotype (22). Subjects with FCHL are at greatly increased risk of CAD, like those with

hyperapobetalipoproteinemia. They too have LDL that differ in composition from LDL of normal individuals.

LDL isolated from individuals with diverse lipoprotein phenotypes differ in their uptake by cultured fibroblasts (3, 4, 15). Furthermore preliminary studies attempting to explore kinetic heterogeneity of LDL in people suggest that differing patterns of heterogeneity are found between individuals classified as having FH, FCHL or hyperapobetalipoproteinemia. For example, Foster, Chait and colleagues found that different kinetic models were needed to adequately describe LDL kinetics in subjects with FH and FCHL (10,11).

Differences in LDL isolated from the same individual at different times. Krauss and colleagues have described a complex pattern of LDL heterogeneity in which up to seven different subclasses of particles can be distinguished on the basis of compositional, flotational and particle size differences (7). These patterns can change in the same individual in response to various environmental manipulations (23).

We have recently published the observation that cholestyramine treatment in the guinea pig results in LDL in which there is an increased proportion of small particles with a decreased cholesterol-to-protein ratio (1). When LDL from treated animals (Rx-LDL) was isolated, radiolabeled and reinjected simultaneously with LDL isolated from chow-fed control animals (Con-LDL), the Rx-LDL had an intrinsically slower clearance than did Con-LDL. Furthermore in a fibroblast cell culture LDL-receptor mediated binding and uptake of Con-LDL was greater than that of Rx-LDL. Similarly, if Con-LDL and Rx-LDL were glucosylated, a procedure which abolishes the ability of LDL to bind to the LDL-receptor, the difference in clearance between Con-LDL and Rx-LDL was abolished. Thus cholestyramine treatment alters the composition and intrinsic metabolic properties of LDL.

We have also shown that bile sequestrant therapy produces similar changes in LDL composition in man (24). In recent studies performed on the metabolic ward, we again documented that colestipol therapy produced LDL that was depleted in cholesterol, smaller in size and more dense. Analytical ultracentrifugation revealed a selective decrease in large LDL particles while small LDL remained (2). Thus colestipol therapy produced a population of LDL that were similar to the LDL found in patients with hyperapobetalipoproteinemia!

To determine if such compositional changes also led to alterations in metabolism we housed patients on the metabolic ward. LDL were isolated from the same subject during a period of bile sequestrant treatment (Rx-LDL) and then, in the absence of treatment (Con-LDL). The two LDL preparations were radiolabeled and simultaneously reinjected into the donor, who had either resumed therapy or, in some cases, remained off therapy.

Nine turnover studies were performed with six human subjects using this basic protocol. Consistent with our results in the guinea pig, the plasma clearance of Rx-LDL was substantially lower in six of eight patients. This was true in several subjects in whom the protocol was repeated, but in reverse order. Numerous control studies made it unlikely that the difference in clearance of the two LDL preparations was due to an experimental artifact (2).

In a similar manner, Eisenberg and colleagues have shown that LDL isolated

from hypertriglyceridemic subjects are relatively small, cholesterol-ester depleted and triglyceride enriched. Such LDL have a decreased uptake by the LDL-receptor of fibroblasts (3, 4). If the hypertriglyceridemia is treated with gemfibrozil, the composition of the LDL reverts toward normal and the uptake of such LDL improves, i.e., this effect is opposite to that noted with cholestyramine treatment above.

In order to explain these findings we have proposed that hepatic LDL-receptor activity plays a major role in modulating LDL composition. We propose that large LDL are preferentially removed by the hepatic LDL receptor, leaving behind in plasma proportionately more small, cholesterol-ester depleted particles. The residual particles in turn have a decreased affinity for the LDL-receptor. In response to cholestyramine therapy, which increases hepatic LDL-receptor activity, larger, more buoyant LDL are removed at a greater rate than smaller LDL. This results in a greater depletion of LDL cholesterol, than of LDL apo B. In contrast, when hepatic LDL-receptor activity is low, as in patients with familial hypercholesterolemia, cholesterol-enriched LDL particles accumulate. In a similar fashion we propose that in patients with hyperapobetalipoproteinemia there is an increased production of LDL. However, LDL-receptor activity is high. The reasons for this are not clear but may be related to alterations in hepatic bile acid or triglyceride metabolism. Consequently, large cholesterol-enriched LDL are preferentially removed, maintaining LDL-cholesterol levels in the "normal" range, but LDL apo B levels are high as small LDL accumulate. Teng et al. have recently presented evidence that LDL production rates are increased in such patients (25).

Differences in LDL isolated from an individual at a single point in time. Several investigators have reported that the physical properties of LDL subpopulations found in some individuals are not homogeneous. As noted above Krauss and colleagues (7) have defined seven different subpopulations of LDL separated on the basis of physical properties. However, with the exception of the recent publication of Teng et al. (25) no one has reported a study looking at metabolic properties of LDL subfractions isolated from the same individual.

As previously discussed, LDL (i.e., d = 1.025 - 1.063 g/ml) isolated from a subject on colestipol therapy (Rx-LDL) had a lower FCR than LDL isolated from the same subject in the absence of therapy (Con-LDL). We have proposed that this was due to the fact that in Rx-LDL there was a depletion of larger, lighter LDL particles and a relative accumulation of smaller LDL particles. We have proposed that the smaller, denser LDL particles have a decreased rate of plasma clearance compared to larger LDL and that it is the shift in particle size from larger to smaller particles that explains why Rx-LDL has a slower clearance compared to Con-LDL.

In the studies noted above, however, we had used whole LDL samples, (i.e., d = 1.025 - 1.063 g/ml). Therefore, to directly test this idea we conducted metabolic studies in five individuals from whom "light" and "heavy" LDL subfractions were actually isolated by ultracentrifugation. We will use the terms "light" and "heavy" to describe the two LDL subfractions isolated with the understanding that these terms refer to the buoyant density of the particles, not to their mass. All subjects were housed on the metabolic ward and consumed an AHA, phase I diet for 5-7 days. One-half to one unit of plasma was obtained by plasmaphoresis and subjects were then started on colestipol HCl (10 grams b.i.d.) to induce expression of hepatic LDL-receptors. On the basis of studies in guinea pigs, we postulated that the light LDL would have a

shorter mean residence time (i.e., an increased rate of plasma clearance) due to greater LDL-receptor mediated clearance. Thus we wanted to inject the two LDL subfractions into the donor at a time the donor expressed maximal hepatic LDL-receptor activity.

For each subject a light LDL subfraction of density = 1.025 to 1.040 g/ml, and a heavy LDL subfraction of d = 1.050 - 1.063 g/ml, were prepared by sequential ultracentrifugation in a fixed angle rotor. Each sample was washed once at each of the limiting densities and all density solutions were monitored with a densitometer. Light and heavy LDL subfractions were labeled with ^{131}I or ^{125}I, respectively, and reinjected into the donor as previously described (26). Plasma samples were obtained at frequent time intervals during the first 48 hours and daily thereafter for two weeks. Urine samples were also obtained as previously described (26). For each time point a whole plasma sample was counted, and plasma decay curves constructed for each tracer. In these pilot studies we did not determine the mass of apo B in individual subfractions, since we did not know if the tracers were representative of the tracee pools separated by the ultracentrifugation step.

For these studies we selected patients with mild hypertriglyceridemia as this made it possible to isolate sufficient amounts of heavy LDL. Several attempts to isolate such LDL (1.050 - 1.063 g/ml) from normal controls failed to provide sufficient mass to perform a turnover study. Subject 1 was a 70-year-old man with longstanding CAD and a type IIb phenotype. Subject 2 was a 63-year-old asymptomatic woman with phenotype IIb. Subject 3 was a 64-year-old man with CAD and a type IV phenotype. Subject 4 was a 46-year-old man with CAD, type IIb phenotype, and a strong family history of hypercholesterolemia but no xanthomas. Subject 5 was a 47-year-old asymptomatic male with a type IV phenotype.

Figure 1 displays the plasma decay and the U/P ratios observed after the simultaneous injection of radioiodinated heavy and light LDL in Subject 1. The whole plasma decay of the light LDL label was clearly faster than that of the heavy. Calculation of the plasma mean residence time (MRT) using a traditional two pool model (27) yielded estimates of 1.88 and 2.11 days for the light and heavy tracers respectively (Table 1). Inspection of the U/P data shows a higher U/P ratio for the light subfraction label, consistent with its faster clearance. When residence times were calculated from this data (the "P/U" ratio in Table 1) values of 1.33 and 1.89 days were found for the light and heavy LDL respectively. However it is also clear that the U/P ratio for neither isotope reached a stable plateau. Furthermore, the slope for the light LDL was much greater than that for the heavy LDL. We have previously reported that the U/P ratios, even for whole LDL, frequently fail to reach a plateau (26) , and suggested that the declining U/P ratios over time reflected kinetic heterogeneity. Berman et al. (9) and Foster et al. (10) have also reported similar findings in some subjects. In fact, the only time we have seen constant U/P ratios was when an iodinated glucosylated-LDL tracer was injected (26). Such a tracer is incapable of interacting with the LDL-receptor. In an analogous situation, constant U/P ratios have been observed in subjects with homozygous FH, who congenitally lack LDL-receptors. This suggests that the metabolic heterogeneity is in part due to interaction with the LDL-receptor, the early appearing peak of the U/P curve being due to binding of LDL to its receptor. The U/P data for the LDL subfractions of Subject 1 suggest that there is metabolic heterogeneity within LDL subfractions, and that there is more heterogeneity in the light LDL tracer than in the heavy. This is also

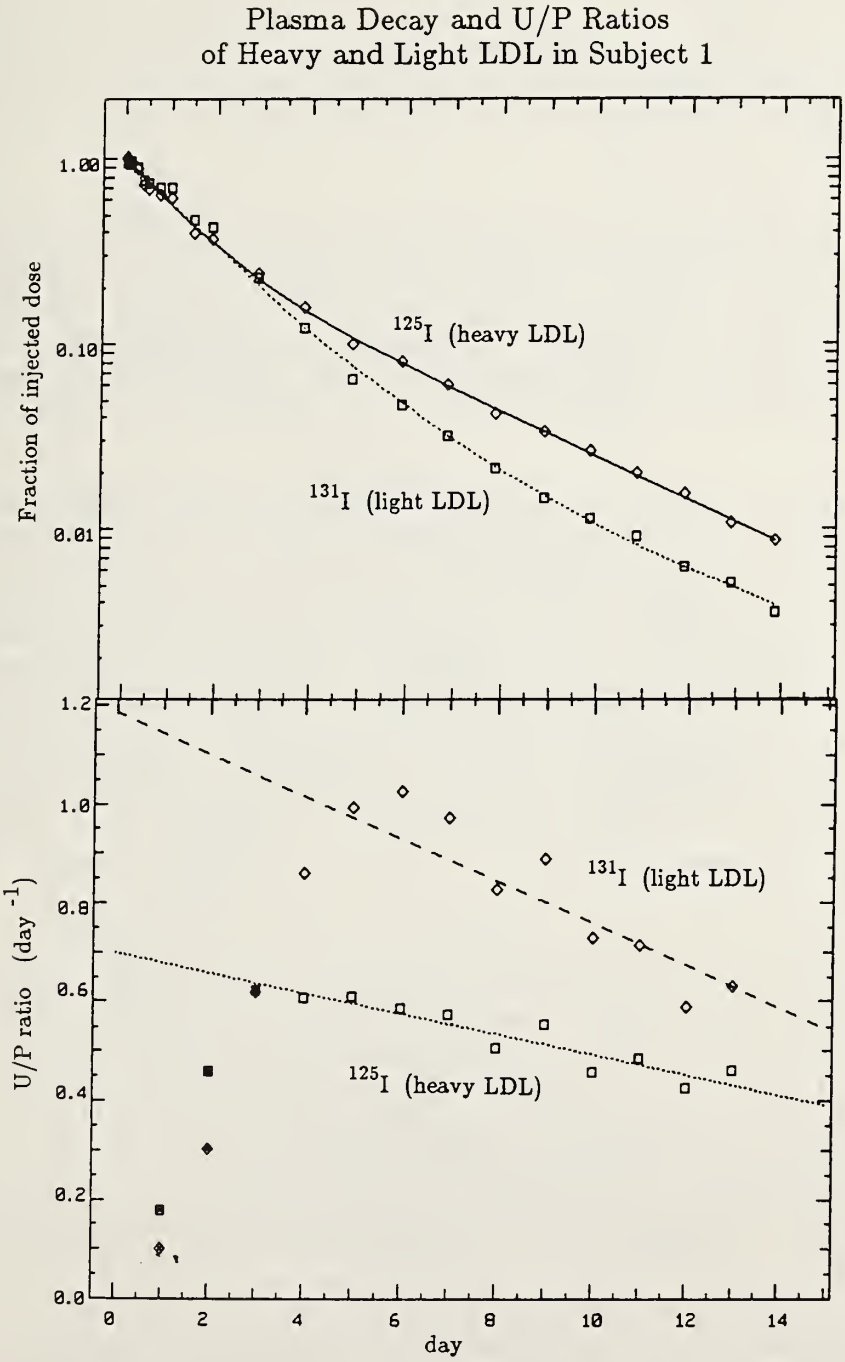

Figure 1. Plasma decay curves (upper panel) and U/P ratios (lower panel) for Subject 1.

consistent with our previous observation in guinea pigs that such light LDL have a greater affinity for the LDL-receptor. To further explore this hypothesis, the two human LDL tracers were injected intravenously into guinea pigs. Again, the light LDL had a more rapid turnover than the heavy. The two labels were then glucosylated and injected into other guinea pigs. The clearance of both tracers from plasma was now greatly retarded and nearly equal, their non-receptor mediated clearances were therefore equal and the difference in clearance rate of the native tracers was due to LDL receptor-mediated processes. These results are consistent with recent observations of Thompson et al. (28).

Inspection of the subfraction data was most revealing. In Figure 2 is shown the plasma disappearance of each tracer in the 1.045 g/ml top and bottom fractions of ultracentrifugally separated plasma. The ^{131}I-light LDL (d = 1.025 - 1.040 g/ml) had a biexponential decay as measured in the 1.045 g/ml top fraction (lower portion of Figure 2). Of great interest was the substantial conversion of ^{131}I into the bottom fraction, seen as the rise in the activity of ^{131}I in the bottom. This indicates the conversion of light LDL into heavy.

In the top panel of Figure 2 is shown similar data following the injection of the ^{125}I-heavy LDL. The plasma disappearance of ^{125}I-heavy LDL from the bottom pool is much less curvilinear than was true for the ^{131}I-light LDL in the top fraction of plasma. This is consistent with the idea that the metabolism of the heavy LDL is less dependent on the LDL receptor than is true for light LDL. Of note also is the substantial amount of ^{125}I-heavy LDL that is observed in the 1.045 top. Approximately 10% of the injected dose was found in the top at the time of first sampling at 10 minutes after injection. A similar pattern was observed in 3 of the 5 individuals: the maximum amount of ^{125}I radioactivity in the top fraction was already present at 10 minutes. This is similar to the observations of Teng et al. (25). In two individuals, however, there was evidence of conversion of heavy to light observed during the three hours after injection.

In order to obtain initial estimates of the residence times of each LDL subfraction label in both the 1.045 g/ml top and bottom "pools" of plasma, we established the preliminary model shown in Figure 3. In this model, L refers to the light LDL pool (d = 1.025 - 1.045 g/ml) and H refers to the heavy LDL pool (d = 1.045 - 1.063 g/ml). T stands for the sampling of radioactivity in the 1.045 g/ml top, and B for that in the 1.045 g/ml bottom fraction of plasma. P represents the measurement of whole plasma radioactivity. Lx is an exchange pool for light LDL and Hx a similar pool for heavy LDL. In the preliminary analysis we included a unidirectional conversion of light LDL to heavy to fit the ^{131}I bottom data. Radioactivity is introduced into both the heavy and light LDL pools.

Initial estimates of mean residence times for the two tracers in the two subfractions (4 estimates) were obtained by estimating only those parameters required to fit an individual curve and performing the four analyses independently. This calculation has the advantage that it allows comparison of the "apparent" removal rates of the two isotopes from the two density ranges in a relatively "model-independent" way. The results are presented as mean residence times in Table 1. To estimate the residence time of the ^{131}I-light tracer in the 1.045 top, an adequate fit was obtained with the portion of the model shown on the left in Figure 4. To obtain estimates of the ^{131}I-light LDL

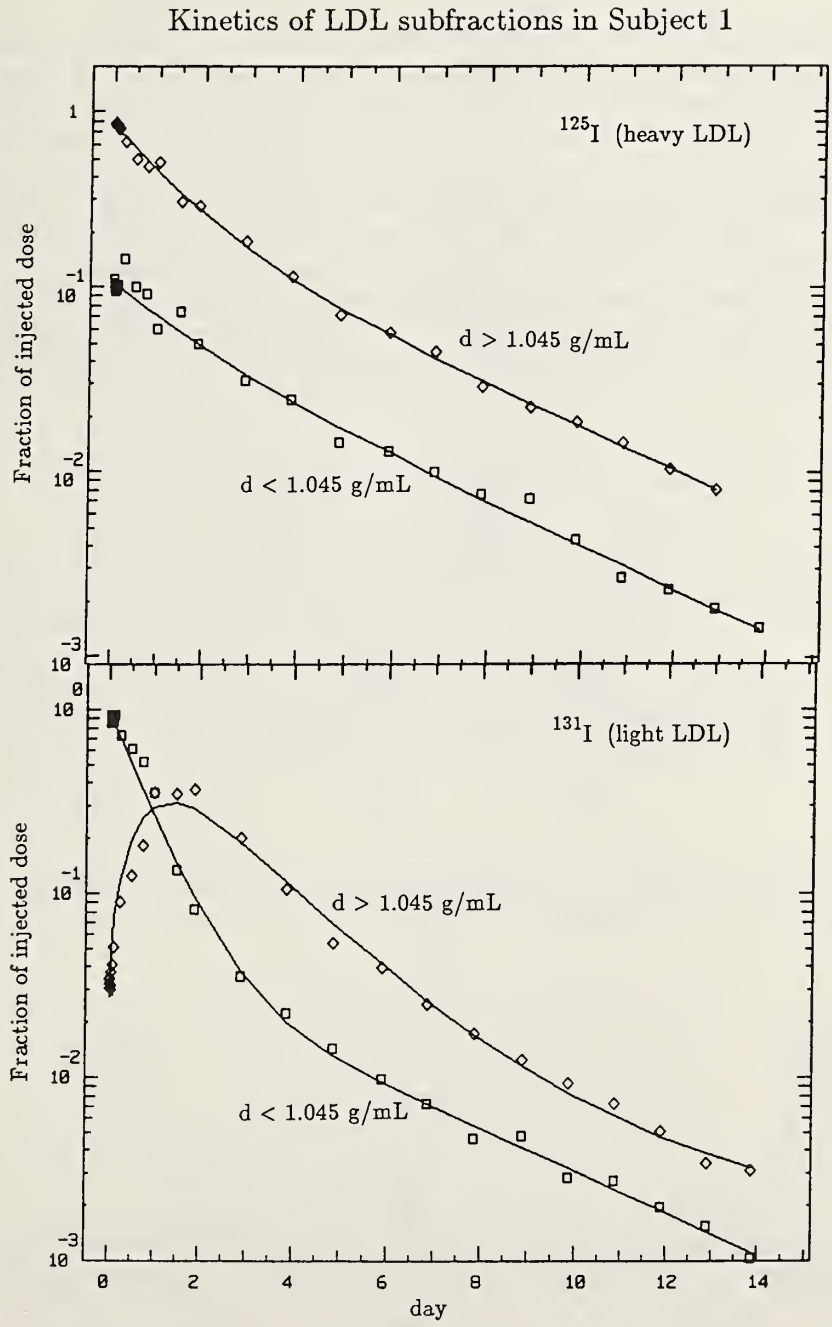

Figure 2. Tracer kinetic data for ^{125}I-labeled heavy LDL (upper panel) and ^{131}I-labeled light LDL (lower panel) for Subject 1. Shown in each panel is the measured activity of the isotope in both the d < 1.045 g/ml top and the d > 1.045 g/ml bottom subfractions.

tracer in the 1.045 bottom, the portion of the model shown on the right in Figure 4 was required. Using these models, estimates for residence times of the ^{131}I-light LDL tracer in the 1.045 g/ml top and bottom fractions of subject 1 were 0.86 and 1.58 days, respectively. Thus, when the light LDL was converted to the heavy LDL pool it took on the metabolic properties of heavy LDL, i.e., a prolonged residence time.

To obtain estimates of the residence times of the ^{125}I-heavy LDL tracer in the heavy LDL pool, the portion of the model shown in Fig. 5 was used. Because the ^{125}I-tracer that was present in the 1.045 g/ml top did not exhibit a rise indicating ongoing conversion from the bottom, we used the same 2-pool model shown on the left of Figure 4. The calculated residence time of the ^{125}I-heavy LDL tracer was 2.07 days in the heavy pool, but 2.72 in the top. However as noted in Table 1, this proved to be the exception. In all of the other four individuals the residence time of the heavy LDL tracer was greater in the heavy pool than when it was converted to the light pool. Thus, upon conversion from the heavy pool into the light pool the ^{125}I-tracer usually took on metabolic properties more akin to that of the light pool, i.e., a faster rate of clearance.

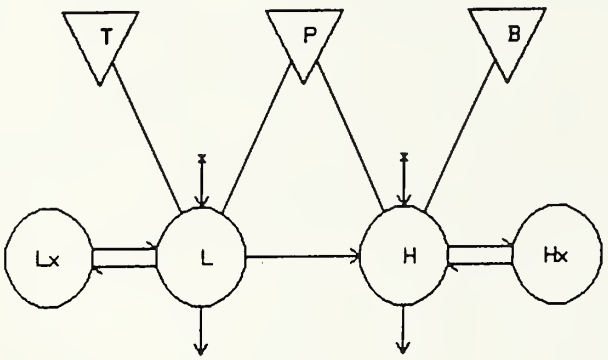

Figure 3. Preliminary model used to calculate mean residence times for both labels in both density subfractions. The model is described in the text.

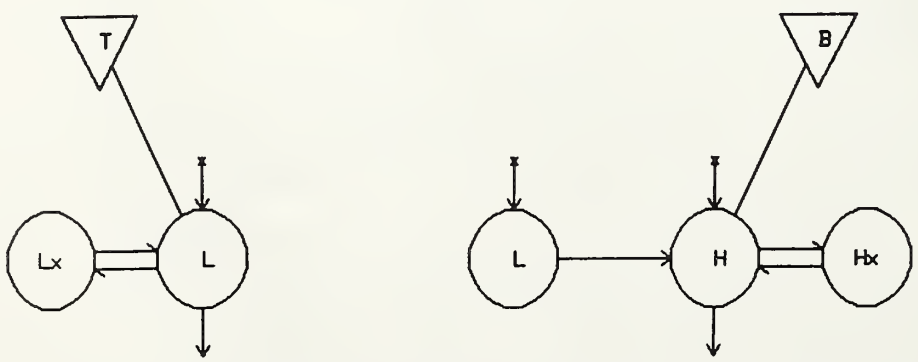

Figure 4. Subsets of the model shown in Figure 3 which were used to calculate mean residence times for ^{131}I in both subfractions and ^{125}I in the 1.045 top. The submodel on the left was used for each isotope as measured in the top. The submodel on the right was used for the ^{131}I-bottom data.

Table 1. Mean Residence Times of Light and Heavy LDL Labels in Whole Plasma and in d 1.045 g/ml Top and Bottom Subfractions

Subject	Phenotype	Fraction Injected	Mean Residence Time (days) P/U	Plasma	Top	Bottom
1	IIb	Light	1.31 ± .07	1.88 ± .04	0.86 ± .02	1.58 ± .44
		Heavy	1.89 ± .15	2.11 ± .05	2.72 ± .10	2.07 ± .08
2	IIb	Light	1.86 ± .11	1.49 ± .04	0.99 ± .03	1.66 ± .14
		Heavy	2.55 ± .20	1.83 ± .04	1.62 ± .06	2.04 ± .07
3	IV	Light	2.01 ± .11	2.33 ± .04	1.34 ± .05	1.54 ± 2.99
		Heavy	2.67 ± .19	2.35 ± .05	1.25 ± .23	2.11 ± .08
4	IIb	Light	1.46 ± .12	2.19 ± .06	1.65 ± .07	2.90 ± 3.13
		Heavy	1.99 ± .22	2.97 ± .07	2.25 ± .10	3.92 ± .11
5	IV	Light	0.93 ± .12	0.94 ± .05	0.63 ± .01	0.96 ± .25
		Heavy	1.22 ± .15	0.95 ± .05	0.64 ± .03	1.28 ± .04

Residence times are shown as estimate ± asymptotic standard error as calculated by SAAM (29). U/P ratios were calculated as daily urinary iodine recovery divided by mean plasma activity during the period of urine accumulation. The average U/P ratio was calculated as the mean of the U/P ratios for all days except the first and the P/U ratio was calculated as the reciprocal of this value. Residence times for plasma, top and bottom were calculated using the models shown above and described in the text.

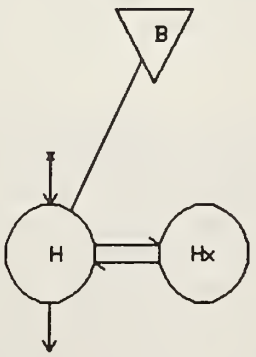

Figure 5. Subset of model shown in Figure 3 used to calculate the mean residence time of ^{125}I in the 1.045 bottom.

It should be appreciated that the residence times of the ^{125}I-heavy LDL in the light LDL pool presented in Table 1 were calculated with the assumption that after the first 10 minute time point there was no further conversion of heavy ^{125}I to light. If there is continued conversion of heavy into light, then the estimates given in Table 1 would be overestimates. It is therefore possible that part of the difference in estimated residence times between the two tracers in the light LDL pool is due to this oversimplication of the model.

In one subject (#3) a significant increase of ^{125}I in the 1.045 top was observed. For this case, introduction of label into the heavy fraction and its subsequent conversion to light LDL had to be included in the modeling.

Studies similar to those described for Subject 1 were performed in four other subjects. Figure 6 shows the whole plasma decay curves of light and heavy LDL for Subjects 2 and 3. These two subjects presented the extremes in differences between plasma curves for the two isotopes. In Subject 2 the light LDL had a residence time of 1.49 days while the heavy LDL had a residence time of 1.83 days. Note that the residence time of the light LDL was again prolonged after it was converted to the heavier pool (0.99 vs. 1.66 days). Conversely, the residence time of the heavy LDL fraction was shortened when it was converted to the top fraction (1.62 vs. 2.04 days). In Subject 3 only a minimal difference was seen in the plasma decay of light and heavy LDL (Fig. 6, lower panel). Yet, the subfraction data again demonstrated that tracers were always cleared more rapidly from the light LDL pool than they were from the heavy LDL pool.

In Subject 4, the whole plasma decay of the heavy LDL was considerably slower than that of the light LDL (residence time of 2.97 vs. 2.19 days). The subfraction data was most interesting. Again, there was substantial conversion of light LDL to heavy (lower panel, Fig. 7). Inspection of the top panel of Fig. 7 indicates that 10 minutes after injection of the ^{125}I-heavy LDL, nearly 70% of the counts were already in the light pool! Furthermore, over the next three hours there was actually a small rise in radioactivity, suggesting the continued transfer of ^{125}I from the bottom to the top. At the same time there was a relative plateau in the initial portion of the ^{125}I-decay curve in the heavy LDL pool. This probably results from transfer of ^{125}I counts back to the bottom.

Subject 5, who had the most severe underlying hypertriglyceridemia, had the fastest absolute clearances of both heavy and light LDL, which were nearly equal. Despite this, the subfraction data again showed that both tracers were cleared more rapidly from the light pool than they were from the bottom pool (Table 1).

Clearly, in all five individuals there was conversion of light LDL into heavy LDL. This is similar to results recently reported by Teng et al. (25). However, in all five patients significant amounts of radioactivity initially introduced in the heavy fraction were observed in the top at the first time point sampled, 10 minutes. One possibility to account for this phenomenon is that it represented a fraction of heavy LDL that was rapidly converted prior to the initial time point. In two individuals there was also evidence for conversion of ^{125}I radioactivity into the top for up to three hours after injection. An alternative explanation is that some of the material originally isolated and labeled with ^{125}I and designated heavy LDL, in fact contained a population of light particles. For each individual, aliquots of the injectate containing the heavy and light LDL tracers were mixed with BSA and spun at density 1.045 g/ml, a procedure identical to the handling of the plasma samples. For the ^{131}I-light LDL tracer 90% or more of the label was found in the 1.045 top. In the case of the ^{125}I-heavy LDL, however, only 75-85% of the counts were in the bottom. However, if the tracers were incubated at room temperature with fresh whole plasma for ten minutes prior to density separation, up to 50% of ^{125}I counts now floated. An example is shown in Table 2 with the tracers prepared from Subject 2.

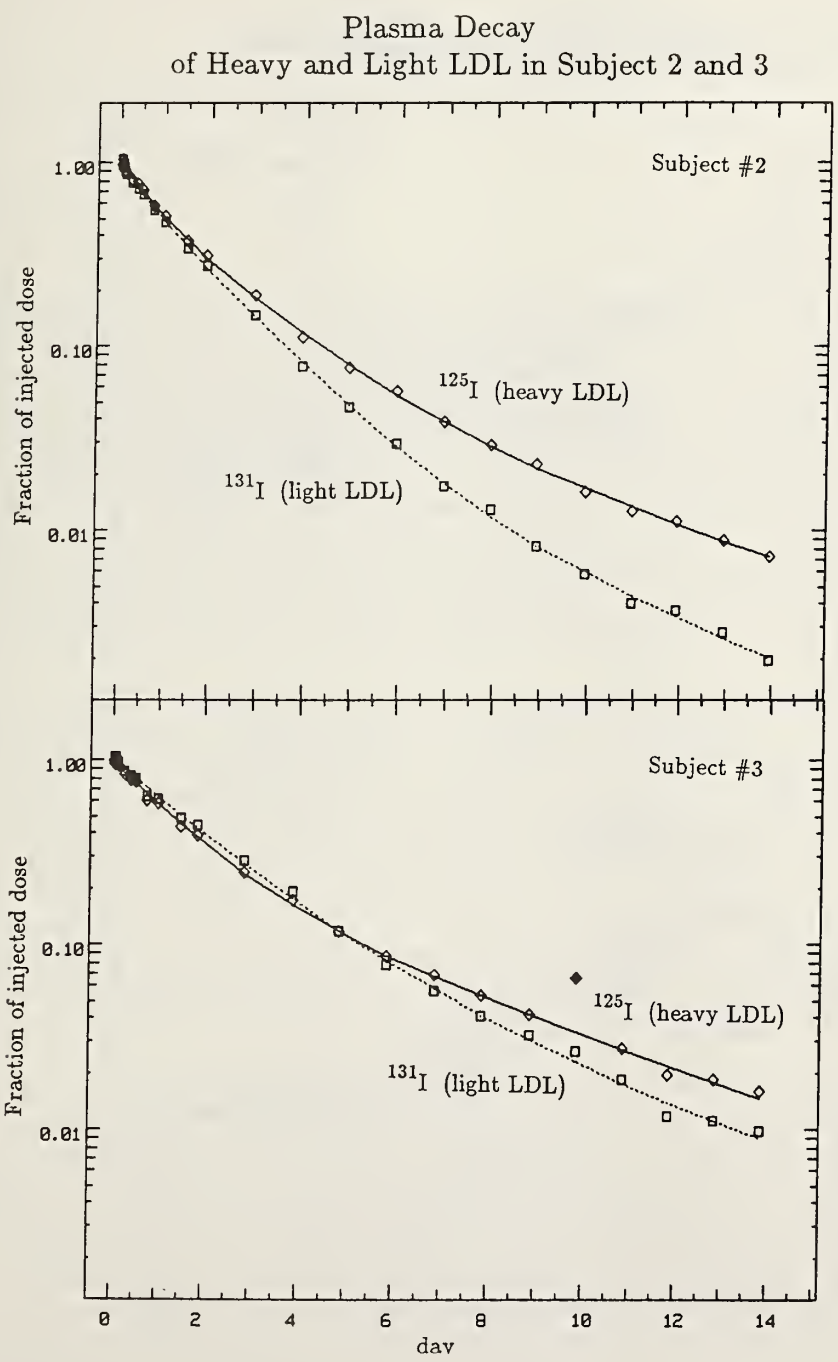

Figure 6. Plasma decay curves for Subject 2 (upper panel) and Subject 3 (lower panel).

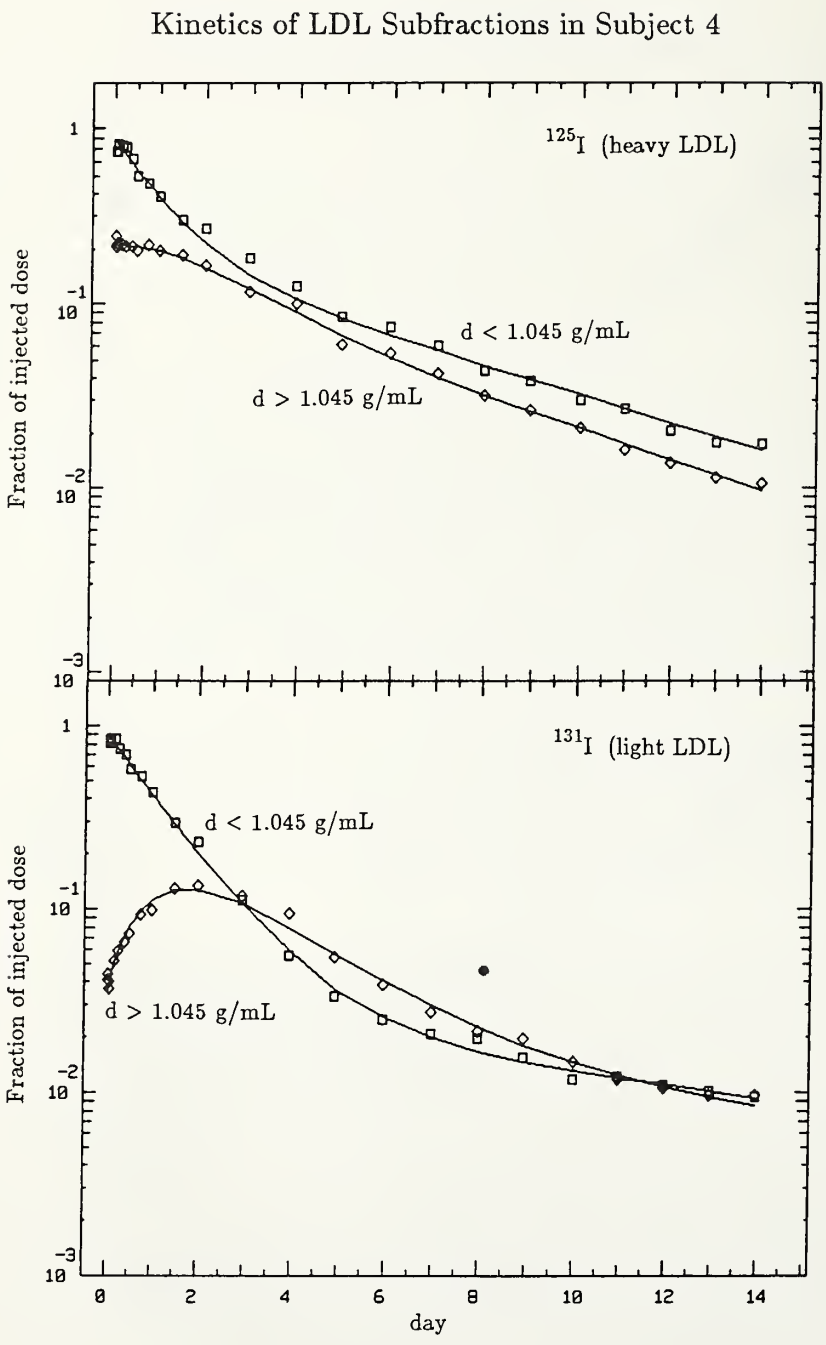

Figure 7. Tracer kinetic data for ^{125}I-labeled heavy LDL (upper panel) and ^{131}I-labeled light LDL (lower panel) for Subject 4. Shown in each panel is the measured activity of the isotope in both the d < 1.045 g/ml top and the d > 1.045 g/ml bottom subfractions.

Table 2. Percent of Radioactivity in 1.045 g/ml Top and Bottom Fractions

		10 minute incubation in albumin	10 minute incubation with plasma	24 hr incubation with plasma
^{131}I-light LDL	Top	91%	97%	87%
	Bottom	9%	3%	13%
^{125}I-heavy LDL	Top	17%	45%	61%
	Bottom	83%	55%	39%

In this experiment the two tracers were incubated with either albumin or plasma for the indicated times and then spun at d 1.045 g/ml. The top and bottom fractions were counted and radioactivity of each tracer expressed as percent of total. Clearly incubation with plasma for even 10 minutes had a profound effect on the heavy LDL, causing a substantial shift in density of many of the particles. Thus, although some of the heavy LDL floated on respinning, it is more likely that incubation with plasma led to the substantial amount of ^{125}I-radioactivity that floated even in the 10 minute plasma sample. In contrast, most of the light LDL tracer remained light, emphasizing the fact that the intravascular conversion of light LDL to dense LDL requires intravascular metabolic processing, such as interaction with lipolytic enzymes.

Finally, we looked at the plasma density distribution of the two LDL tracers following their injection. An example for Subject 2 is shown in Figure 8. Plasma samples obtained 10 minutes, 9 hours and 72 hours after injection were subjected to density gradient ultracentrifugation and the counts in each fraction expressed as percent of total recovered for that sample. It can be seen that in the counts remaining there is a shift in modal density of the ^{131}I-light LDL to a heavier fraction, while that of the heavy remained relatively constant. Since this data represents density of counts <u>remaining</u>, it is compatible with the interpretation that over time the lighter particles are removed more rapidly, while the slower, residual particles, which are more dense, account for an increasing proportion of residual particles.

In order to further study the degree of LDL kinetic heterogeneity implied by the data, all data were analyzed using multicompartmental modeling. The model shown in Figure 9 was first considered. In this model, plasma LDL is comprised of two kinetically distinct compartments, one (F) being removed more rapidly than the other (S). Bidirectional interconversion of particles between these two compartments is allowed for. Irreversible removal of all LDL isotope is into an iodide pool (I) which is apparent as a delay before its subsequent appearance in urine (U). It is further allowed that a fraction of each LDL compartment may be measured in both the 1.045 top (T) and the 1.045 bottom (B). Measured plasma data (P) was the sum of the top and bottom activities. This model proved to be inadequate in describing all data simultaneously because of the difference in how fast the two labels were observed to be removed from the 1.045 bottom (Table 1). The conversion of ^{131}I from the top to the bottom couldn't be into a pool which turned over as slowly as one consistent with the ^{131}I bottom data and the urine data. Thus, further kinetic heterogeneity <u>within</u> the heavy density range was implied.

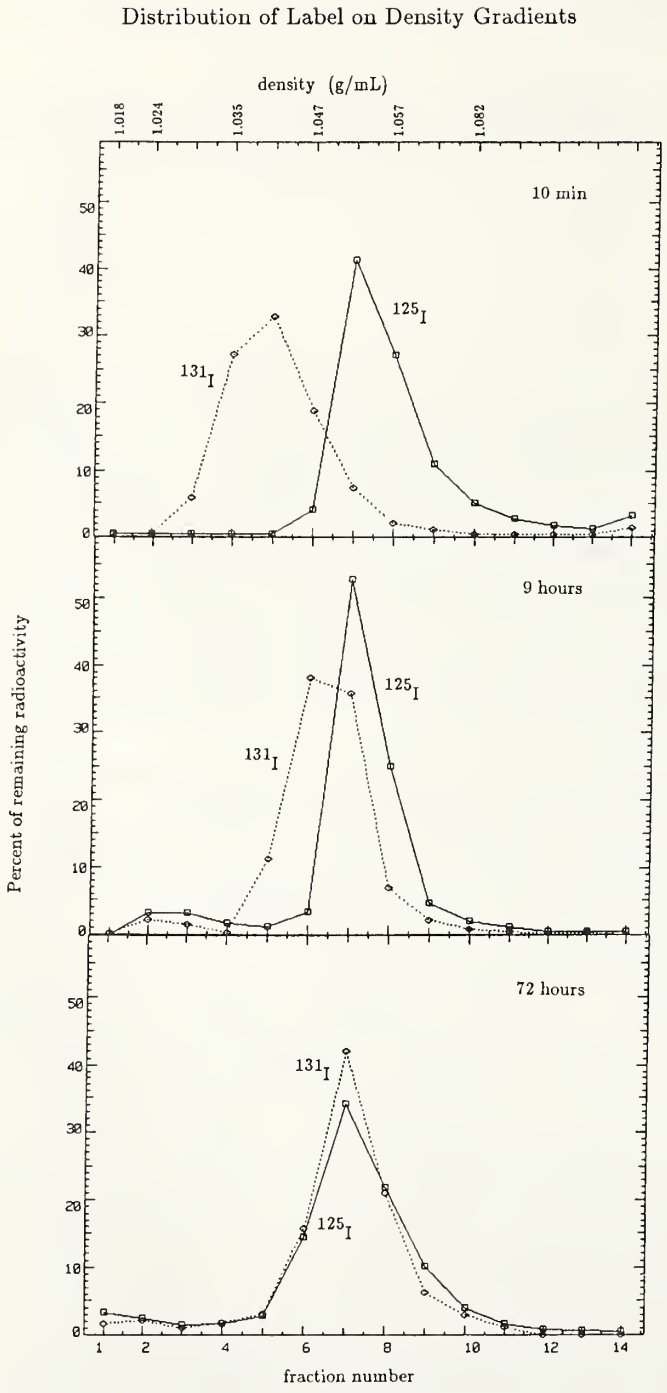

Figure 8. Distibution of ^{125}I and ^{131}I as measured on density gradients at 10 minutes (upper panel), 9 hours (moddle panel) and 3 days (lower panel) after injection.

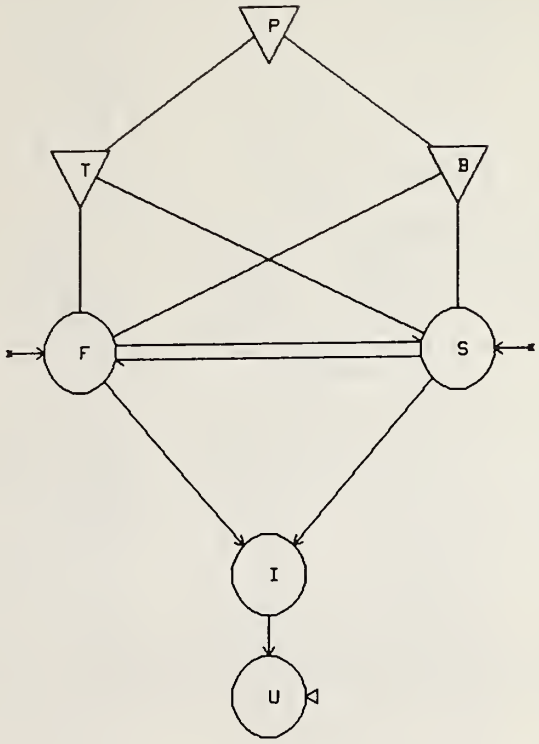

Figure 9. Preliminary multicompartmental model used to simultaneously analyze all kinetic data. Compartments are: P, measured plasma radioactivity; T, radioactivity measured in the 1.045 g/ml top; B, radioactivity measured in the 1.045 g/ml bottom; F, "fast" LDL; S, "slow" LDL; I, iodine; U, urine. Starred arrows indicate sites of introduction of tracer into the system.

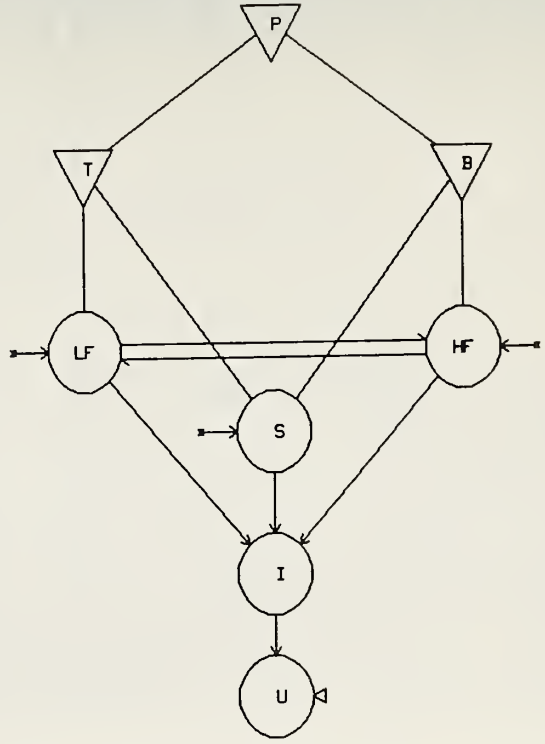

Figure 10. Final multicompartmental model used to analyze all kinetic data. Compartments are: P, measured plasma radioactivity: T, radioactivity measured in the 1.045 g/ml top; B, radioactivity measured in the 1.045 g/ml bottom; LF, "fast" LDL measured in the 1.045 top; HF, "fast" LDL measured in the 1.045 bottom; S, "slow" LDL: I, iodine pool; U, urine. Starred arrows indicate sites of introduction of tracer into the system.

The model shown in Figure 10 was then tested. In this model, the fast pool of Figure 9 has been divided into two pools, one (LF) measured in the 1.045 top and the other (HF) measured in the 1.045 bottom. The interconversions of label between the light and heavy fractions are between these compartments. The slow LDL moiety (S) is retained, again with a fraction measured in the top and the remainder in the bottom. All LDL pools have direct removal from plasma. This model gave a good fit to all data and the model parameters giving the best fits are presented in Table 3. Fractional conversion and removal rates were constrained to be the same for both isotopes for a single subject. Indeed, the only difference between the two labels was the initial distribution among the three LDL compartments. For all subjects, a greater fraction of the ^{125}I was found in the slow pool than for ^{131}I. In all subjects except #4, a greater fraction of the slow pool was observed in the heavy fraction. These observations suggest that the distribution of slow LDL is shifted to a denser population when compared to that for the fast particles. In 4 of the 5 subjects, the direct removal of light fast LDL was faster than for heavy fast LDL. In one subject (#3) these rates were equal. The removal rates for heavy, fast LDL were about three times faster than those for slow LDL. The removal rates for slow LDL are somewhat lower than most of those reported by Foster et al. (10) and Chait et al. (11) for the slower of their two LDL compartments (models B and C in their papers) but in the same general range. On the other hand, the removal rates for the heavy, fast LDL in the present study are greater than most of those for the slower pool in Foster et al. (10). This suggests that the two pools proposed by Foster (10) and Goebel et al. (9) correspond to pool S and a lumping of pools LF and HF of the present model.

The source of pool S could not be determined from the present data. Specifically, the possibility of it being a product of LF or HF could not be ruled out. It should be noted that the model in figure 10 does not show any exchange pools for any of the LDL species. No exchange pools were necessary to give adequate fits to the turnover curves. It is possible, however, that some or all of slow components of the curves which were attributed to slow LDL could be explained by exchange. The implications of this and its consistency with the urinary data is currently under investigation.

No steady state calculations (production and transport rates) are presented here and no inputs of endogenous LDL are indicated in the model. Mass determinations were not made and any allocation of mass among the LDL pools would have to be based on the initial distribution of label. However, LDL isolated for labeling was obtained during a different steady state than that during which the turnovers were performed. Thus, even if the distributions of label shown in Table 3 represented the distribution of cold LDL, this distribution would correspond to the control state while the conversion and removal rates shown in Table 3 correspond to those during colestipol treatment.

It is probable that all LDL pools shown in this (and other) compartmental models of LDL metabolism are still "lumped" systems. Our present model suggests an overlap in density of kinetically distinct LDL subspecies, particles which must therefore be separated by criteria other than density to fully explore their metabolism and composition.

Table 3. Estimated Initial Isotope Distributions and Model Parameters

Subject	fraction of $^{131}I_T$ initially in S	fraction of ^{125}I initially in S	fraction of ^{131}I excluding that in S initially in LF	fraction of ^{125}I excluding that in S initially in HF	fraction of S measured in 1.045 top	$L_{I,LF}$	$L_{I,HF}$	$L_{I,S}$	$L_{HF,LF}$	$L_{LF,HF}$	$L_{U,I}$
1	0.033±0.011	0.064±0.020	1.00±0.01	0.89±0.01	0.26±0.01	0.64±0.02	0.46±0.02	0.15±0.02	0.51±0.02	0.10±0.01	0.82±0.03
2	0.010±0.005	0.064±0.022	0.98±0.01	0.61±0.01	0.40±0.02	0.71±0.03	0.49±0.03	0.15±0.02	0.33±0.02	0.09±0.02	0.63±0.02
3	0.053±0.015	0.089±0.024	1.00±0.01	0.81±0.01	0.30±0.01	0.46±0.01	0.46±0.02	0.13±0.02	0.18±0.01	0.05±0.01	1.34±0.08
4	0.098±0.021	0.271±0.045	1.00±0.01	0.15±0.02	0.64±0.02	0.67±0.03	0.45±0.09	0.16±0.01	0.17±0.01	0.00±0.09	0.72±0.03
5	0.051±0.003	0.080±0.005	0.87±0.01	0.79±0.01	0.35±0.01	1.24±0.07	1.17±0.04	0.26±0.01	0.77±0.07	0.00±0.03	1.33±0.04

All parameters are for the model in figure 10. Each value shown is the estimate ± its asymptotic standard deviation as calculated by SAAM (29). The fractional transfer coefficient $L_{I,J}$ is the fraction of compartment J transferred to compartment I per day.

Summary

In this paper we have reviewed evidence that heterogeneity exists in LDL at all three levels discussed in the introduction: LDL differ between individuals, they differ in the same person during different environmental or pharmacologic interventions and even within the same LDL sample, subpopulations of LDL differ in compositional and metabolic properties.

We have proposed that part of the heterogeneity in LDL composition is due to the level of hepatic LDL-receptor activity. Lighter, more buoyant LDL bind to the LDL-receptor with higher affinity. In response to increased hepatic LDL-receptor activity, large, lighter LDL are preferentially removed, leaving an LDL that is now relatively enriched in a heavy, cholesterol depleted LDL subpopulation. In turn, suppression of LDL-receptor activity leads to a relative increase in particles that are larger, more buoyant and cholesterol enriched. Obviously many other factors are involved which our studies have not yet addressed. These include such fundamentally important issues as the possibility that LDL subpopulations have diverse origins and rates of formation and differing abilities to interact with transfer and exchange proteins that profoundly affect LDL composition. In addition our studies have not yet addressed the possibility that in vivo differences in metabolism of light and dense LDL could be mediated in part by the presence of differing amounts of apo E. Obviously there is much to learn, including the fundamental question of whether or not this heterogeneity is of clinical importance.

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Regulation of the Metabolism of Apolipoprotein B Containing Lipoproteins by Apolipoprotein E and the Low Density Lipoprotein and Remnant Lipoprotein Receptors

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Introduction

Apolipoprotein B (apoB) and apoB containing lipoproteins are positive risk factors for the development of atherosclerosis (1-3). The metabolism of these lipoprotein particles is a complex process involving synthesis and secretion of apoB containing lipoproteins, interconversion of different species of apoB containing particles in plasma, and catabolism of these lipoprotein particles (4). We will concentrate on the role of the different isoforms of apoE and of the LDL and lipoprotein remnant receptors in regulating the metabolism of apoB containing lipoprotein particles.

ApoB containing lipoproteins are secreted by the intestine and liver as triglyceride rich particles. They are then metabolized to triglyceride poor, cholesterol rich remnants by the hydrolysis of triglycerides by lipoprotein lipase. The intestinal remnants are then directly catabolized while the hepatic remnants are either directly catabolized or converted on to LDL (4). ApoE and both the LDL and lipoprotein remnant receptors play pivotal roles in the regulation of metabolism of these apoB containing lipoprotein remnants. In order to investigate the metabolism of these particles, apoB-48 can be used as a marker for intestinal particles while apoB-100 is primarily a marker for hepatic particles (5). Intestinal mucosal cells contain apoB-100 mRNA (6), but current evidence suggests that most of the circulating apoB-100 in adults is synthesized by the liver (7).

The role of the LDL receptor in the catabolism of LDL, and mutations of this receptor which are associated with elevated LDL apoB and cholesterol levels are well known and will not be dealt with (8). What is not as well understood is the function of the LDL and lipoprotein remnant receptors in the catabolism of lipoprotein remnants, and the role apoE has in modulating the catabolism of remnants by these receptors. In addition, apoE is genetically polymorphic and the mechanism by which these polymorphic forms alter the *in vivo* metabolism of apoB containing lipoprotein particles is poorly understood.

ApoE is a genetically polymorphic protein in humans (9-11). There are three common alleles in humans designated ε2, ε3, and ε4. This results in three common homozygous and three common heterozygous genotypes. Phenotypes, corresponding to these six genotypes, can be determined from isoelectric focusing patterns of apoE in plasma. The phenotypes of the homozygous genotypes are designated E2, E3, and E4, while the phenotypes of the heterozygous genotypes are E2/3, E2/4, and E3/4. [It is not really appropriate to refer to the E2 phenotype (this also applies to the E3 and E4 phenotypes) as an E2/2 phenotype, as is commonly done in the literature (12), because this strongly implies that this phenotype results from the homozygous ε2/ε2 genotype. The E2 phenotype is actually present in both the ε2/ε2 genotype and the rare heterozygous ε2/εnull genotype, and therefore the designation of the apoE phenotype should not directly infer a homozygous apoE genotype.]

The E3 phenotype is the most common apoE phenotype (10,11) and will be used as the standard to compare the other E phenotypes to. Epidemiologic studies have indicated that individuals with an E2 phenotype have elevated levels of lipoprotein remnants and decreased levels of LDL cholesterol and apoB (13). Subjects with an E2/3 phenotype were also noted to have decreased LDL cholesterol levels and a decreased incidence of myocardial infarctions (see (14) for review). In contrast, individuals with E4 or E3/4 phenotypes had increased levels of LDL cholesterol and apoB, and in some studies an increased incidence of myocardial infarctions (14). Therefore, the E2, E2/3, E4, and E3/4 phenotypes all lead to altered levels of apolipoproteins and lipoproteins and/or incidence of myocardial infaction. In contrast to the epidemiologic studies, in vitro apoE binding studies indicated that only apoE2 has altered receptor binding properties when compard to apoE3, while apoE4 binds normally to cell membrane receptors (15).

In Vivo Metabolism of the Genetic Polymorphic Forms of ApoE in Humans

The following investigations were performed to determine the in vivo metabolism of apoE in humans (16-19). ApoE was isolated from subjects with either an E2, E3, or E4 phenotype. Plasma was obtained, VLDL was isolated by ultracentrifugation and delipidated, and apoE was isolated by heparin affinity and gel permeation chromatography. The isolated apoE was radioiodinated by the iodine monochloride method, reassociated with lipoproteins in whole plasma, the apoE reassociated with lipoproteins was isolated by ultracentrifugation, and this was dialyzed and injected into study subjects. Study subjects were either normolipidemic or hyperlipidemic healthy individuals, on no drugs known to affect lipid and lipoprotein metabolism, and eating an ad lib diet of normal composition. Ten days before the injection of the radiolabeled apoE, the subjects were admitted to the metabolic ward of the NIH Clinical Center and placed on a diet of 42% fat, 42% carbohydrate, 16% protein, 200 mg cholesterol/1000 kcal, and a polyunsaturated to saturated fat ratio of 0.2. Following injection, blood samples were obtained periodically over a seven day period. The distribution of the labeled apoE in plasma lipoprotein subfractions at the first time point was determined following separation of lipoproteins by

ultracentrifugation, and the plasma residence time (residence time = 1/fractional catabolic rate) of apoE was determined from the plasma decay curve utilizing computer assisted curve fitting with the SAAM 29 modeling program.

The distributions in lipoprotein subfractions of radiolabeled apoE2, apoE3, and apoE4 were determined in normolipidemic subjects with an E3 phenotype (17-19). By doing this, other variables were controlled for and the only variable for alterations in the lipoprotein distribution was the isoform of apoE. In this way we were able to determine that, compared to apoE3, less apoE2 was bound to VLDL and more to HDL while more apoE4 was bound to VLDL and less to HDL. Therefore, there was an altered distribution of binding to lipoproteins for both apoE2 and apoE4, but in oppposite directions when compared to apoE3.

When the catabolic rates for apoE2, apoE3, and apoE4 in normolipidemic subjects with an E3 phenotype were compared, the apoE2 had a fractional catabolic rate slower than apoE3 while apoE4 had a markedly increased fractional catabolic rate (17-19). Therefore, compared to apoE3, both apoE2 and apoE4 have altered catabolic rates but once again in opposite directions. From this, we conclude that both apoE2 and apoE4 are metabolically different than apoE3, but in opposite directions.

How can one then explain the differences between the in vitro binding studies, in which only apoE2 has altered binding properties to receptors compared to apoE3 (15), and the epidemiologic and in vivo metabolic studies in which both apoE2 and apoE4 are altered compared to apoE3 (14, 16-19)? One possible explanation is that the in vitro binding studies are measuring only one function of apoE, and therefore, do not predict the full in vivo metabolic consequence of apoE. Another possible explanation is that with the absence of cyteine residues in apoE4, it is unable to form disulfides with other proteins. ApoE3 can form disulfides with proteins containing free sulfhydryls, and it is likely that these apoE3 containing mixed disulfides do not readily exchange between lipoprotein particles (20). In this case, the portion of the apoE3 on HDL that is complexed could not exchange onto newly secreted triglyceride rich lipoprotein particles and would be metabolically inactive. On the other hand, all of the apoE4 would be free to exchange onto these newly secreted particles. This would result in an increased distribution of apoE4 on VLDL, and even with a normal binding affinity for receptors, the increased amount of apoE4 on VLDL could result in the altered metabolism that has been demonstrated. A limitation of the second explanation is that the rapid catabolism of apoE4 results in decreased plasma levels of apoE4 (19). Therefore, even with the increased fractional distribution of apoE4 on VLDL, the decreased absolute plasma concentration of apoE4 results in the total mass of apoE4 on VLDL being similar to the total mass of apoE3. This area is currently under investigation in our laboratory and should be able to be resolved with kinetic studies of chemically modified apoE (21).

In Vivo Metabolism of LDL in Subjects with Altered ApoE Phenotypes

The kinetics of metabolism of chylomicrons, VLDL, and their respective remnants in subjects with altered apoE phenotypes have been investigated. Previous studies have demonstrated that individuals with type III hyperlipoproteinemia and an E2 phenotype have a decreased fractional catabolic rate for remnants of both chylomicrons and VLDL (22-25). In a subject with an Enull phenotype (no apoE), a qualitatively similar but quantitatively greater block in the catabolism of these remnants was determined (26). It is therefore clear, that individuals with either an E2 phenotype or no apoE have a significant block in the catabolism of remnants of triglyceride rich lipoprotein particles which is a result of the functionally abnormal or absent apoE. There is little published data, though, from which to determine the kinetic etiology of the markedly decreased LDL cholesterol and apoB concentrations in these subjects.

In order to investigate LDL metabolism in these individuals in detail, in vivo radiolabeled LDL metabolic studies were performed in subjects with different apoE phenotypes (27-28). LDL were isolated from normolipidemic E3 subjects and from patients with altered forms of apoE, i.e. normolipidemic and hyperlipidemic E2 subjects and a patient with an Enull phenotype. Radiolabeled LDL from an E3 subject and a subject with an altered form of apoE were injected into both types of subjects. The catabolism of the LDL from the two types of subjects were then directly compared in each subject, and the kinetics of metabolism of autologous LDL in the subject with the altered form of apoE was compared to autologous LDL metabolism in the E3 subject.

The results of the LDL metabolic studies in all three types of subjects, normlipidiemic and hyperlipoproteinemic E2 subjects and the Enull subject, were qualitatively similar (27-28). When comparing the catabolism of LDL from the control E3 subjects with the study subjects, the LDL from the subjects with altered forms of apoE were catabolized at a slower rate than the E3 LDL. When comparing the production and catabolic rates of autologous LDL in the study subjects with autologous LDL in the E3 control subjects, all of the subjects with altered forms of apoE had both markedly decreased LDL apoB production rates and increased fractional catabolic rates. From these studies we conclude the following: 1) The LDL formed by these subjects with altered forms of apoE are metabolically abnormal with a decreased fractional catabolic rate both in normal control and study subjects. 2) The rate of production of LDL in these subjects with abnormalities in their apoE is markedly decreased. 3) There is a significant upregulation in the fractional rate of catabolism of LDL in these subjects with dysfunctional or absent apoE.

Regulation of the Metabolism of Remnants of Intestinal and Hepatic Triglyceride Rich Lipoproteins by ApoE and the LDL and Lipoprotein Remnant Receptors

The above results have demonstrated that the common genetically determined forms of apoE are metabolized at different rates, and that

the isoform of apoE present in an individual can have profound effects on the modulation of LDL metabolism. In order to gain additional insights into the regulation of the metabolism of apoB containing lipoprotein particles, studies were performed to investigate the role of the LDL and lipoprotein remnant receptors in the catabolism of remnants of both intestinal and hepatic triglyceride rich lipoprotein particles. Subjects with apoE deficiency have extremely elevated circulating plasma concentrations of these remnant particles (26). Therefore, VLDL ($d<1.006$ g/ml) containing intestinal and hepatic lipoprotein remnants were isolated from a subject with apoE deficiency. These remnant particles were radiolabeled and injected into the Enull patient (26), patients with homozygous familial hypercholesterolemia (HFH) (29), and control subjects with an E3 phenotype. The rate of direct catabolism from the VLDL density range or further metabolism on to IDL and LDL was quantitated utilizing apoB-48 and apoB-100 as markers for intestinal and hepatic remnants, respectively.

In the normal E3 subjects, the apoB-48 containing intestinal remnants were directly catabolized from the VLDL density range at an extremely rapid rate with no conversion on to IDL or LDL (26, 29). The majority of the apoB-100 hepatic remnants were also directly catabolized from VLDL but at a slower rate then the intestinal remnants while 15% of the hepatic remnants were converted on to IDL and LDL. In the Enull subject (26), there was an extremely slow direct catabolism of both intestinal and hepatic remnants from VLDL with virtually no conversion of either remnant on to IDL or LDL. Therefore, apoE is necessary for the direct catabolism of both intestinal and hepatic remnants from VLDL, and for the conversion of hepatic remnants on to IDL and LDL.

The results were quite different in two subjects with HFH compared to the E3 control or Enull subjects (29). One subject was receptor defective with 14% of normal LDL receptor activity while the other subject had less than 5% of normal LDL receptor activity. The results were very similar for both subjects and will be described together. Compared to the E3 subjects, the HFH subjects also directly catabolized apoE-48 intestinal remnants from the VLDL density range at an extremely rapid rate with no conversion on to IDL or LDL. Therefore, an intact LDL receptor is not necessary for the catabolism of intestinal remnants. In contrast to the intestinal remnants, hepatic remnants were catabolized differently in the individuals with HFH than in the normal controls. A much smaller percentage of the hepatic remnants were rapidly catabolized directly from VLDL with 50% of these remnants being converted on to IDL and LDL in the individuals with HFH compared to 15% in the normal subjects. As expected, the resulting LDL particles were then very slowly catabolized in the HFH subjects. Therefore, the LDL receptor is necessary for the rapid direct catabolism of a portion of hepatic remnants from VLDL because in the absence of LDL receptors, fewer hepatic remnants are directly catabolized. In addition, another receptor is involved in the catabolism of these hepatic remnants since 50% of the remnants were rapidly catabolized in the absence of LDL receptors. We conclude that the rapid direct catabolism of hepatic remnants from VLDL is an apoE requiring process involving at least two receptors, the LDL receptor and a second receptor which is likely to be the lipoprotein remnant receptor.

Pathways for the Metabolism of Intestinal and Hepatic Lipoprotein Particles

Fig. 1 is a schematic representation of our proposed metabolic pathways for apoB containing lipoproteins in humans with an E3 phenotype based on the above studies. Triglyceride rich lipoproteins containing apoB-48 are secreted from intestinal mucosal cells, and following secretion the triglycerides are removed by the action of lipoprotein lipase. The triglyceride poor cholesterol rich intestinal remnant particles are then rapidly removed from plasma by the liver via an apoE mediated process by the lipoprotein remnant receptor. The liver also secretes triglyceride rich lipoproteins containing apoB-100. The triglycerides are removed from these hepatic VLDL particles by lipoprotein lipase, and the resulting cholesterol enriched remnants are further metabolized by 3 different apoE mediated processes. 1) They can be directly catabolized by the lipoprotein remnant receptor, 2) they can be directly catabolized by the LDL receptor, or 3) they can be converted on to LDL by a poorly described process that may involve hepatic triglyceride lipase. The resulting LDL is then catabolized by the LDL receptor.

Fig. 2, E2 phenotype panel, depicts the metabolism of these apoB containing lipoprotein particles in subjects with an E2 phenotype who have metabolically defective apoE. In these individuals, the intestine secretes chylomicrons, the triglycerides are hydrolyzed by lipoprotein lipase, and the chylomicron remnants increase in concentration because they can not be efficiently removed by the liver in the absence of a metabolically active apoE. The liver secretes VLDL, the triglycerides are removed but the VLDL remnants accumulate because they can not be efficiently catabolized from plasma by the remnant or LDL receptors and are only slowly converted on to LDL in the presence of the metabolically defective apoE2. Because of the decreased transport of cholesterol into hepatocytes by the apoE mediated catabolism of remnant particles, the LDL receptor is upregulated. The upregulated LDL receptors and the decreased rate of conversion of VLDL remnants on to LDL result in the LDL levels being decreased in E2 subjects. If they have an additional abnormality that results in an increased VLDL production rate, the presence of apoE2 leads to the expression of the hyperlipidemia as type III hyperlipoproteinemia.

In individuals with an E4 phenotype, we propose that the altered metabolism of apoB containing lipoproteins (Fig. 2, E4 phenotype) is the opposite of that in E2 individuals since the *in vivo* metabolic abnormalities of apoE4 are the opposite of those for apoE2. In this case, chylomicrons are secreted by intestinal mucosal cells, the triglycerides are hydrolyzed, and the chylomicron remnants are bound and degraded by the liver more rapidly than in normal E3 subjects. The liver secretes VLDL, the triglycerides are hydrolyzed by lipoprotein lipase and the resulting remnants are then either catabolized by the remnant receptor at an increased rate or further cetabolized on to LDL at an increased rate. Because of the increased rate of cholesterol delivery to the liver resulting from the rapid rate of degradation of chylomicron and VLDL remnants by the remnant receptor, the LDL receptor is down regulated. In these E4 subjects, then, the LDL levels will be

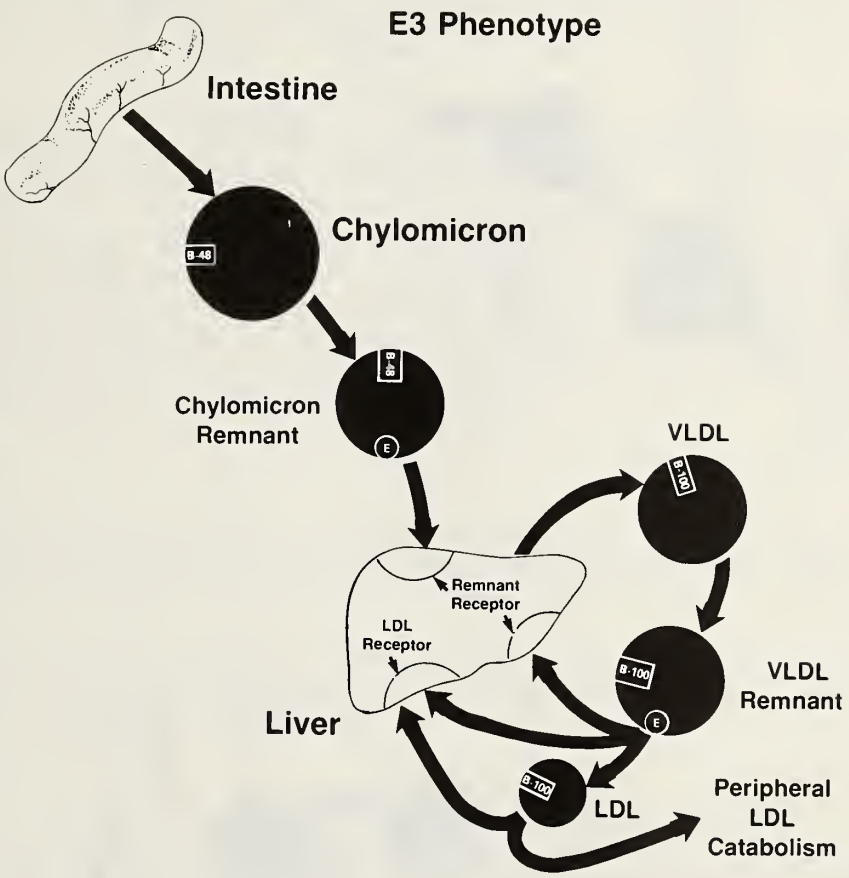
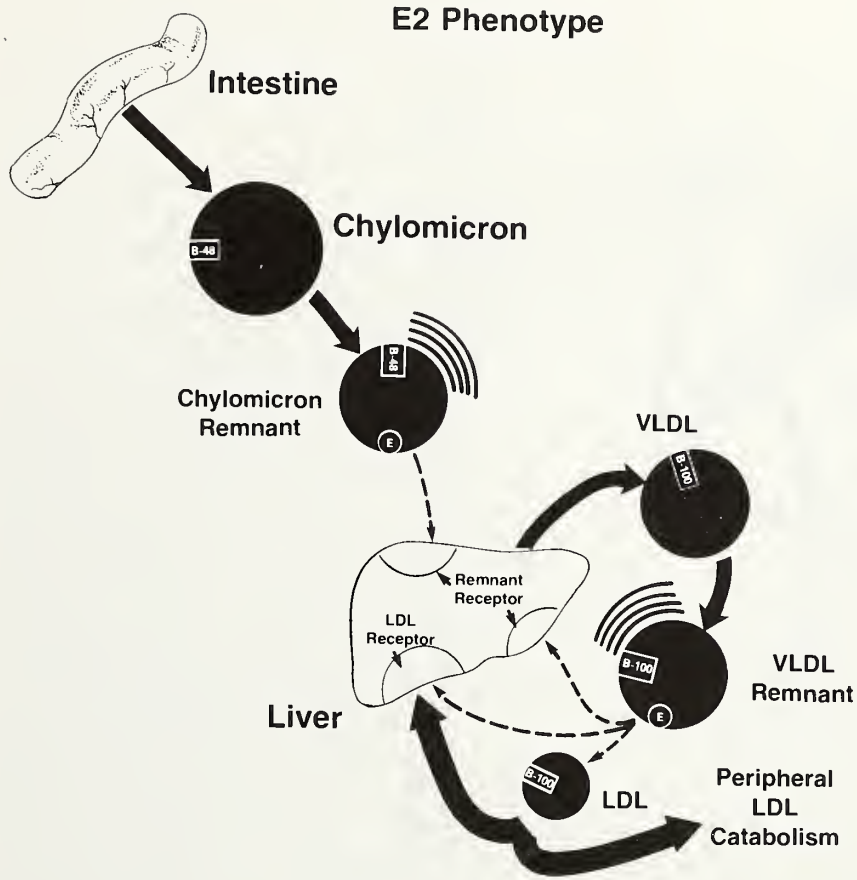


Figure 1. Schematic representation of the metabolism of apolipoprotein B containing lipoproteins in normal humans with an E3 phenotype. See the text for details.



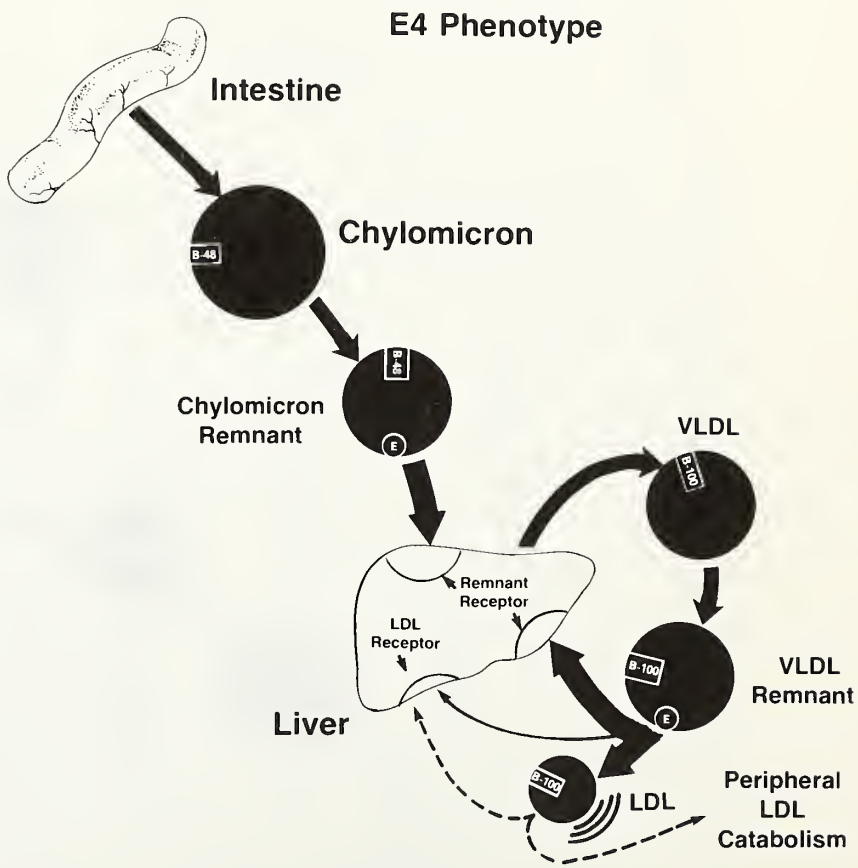


Figure 2. Schematic representation of the metabolism of apolipoprotein B containing lipoproteins in humans with an E2 phenotype or an E4 phenotype. The size of the arrows indicate the rate of the conversion or degradation of the lipoprotein particles while accent marks on a lipoprotein particle indicates the particle is increased in concentration in that phenotype compared to normal E3 subjects. See the text for details.

elevated because of both an increased rate of conversion of VLDL remnants on to LDL and a down regulation of LDL receptor mediated catabolism of LDL. In addition, the direct catabolism of VLDL remnants by the LDL receptor will be decreased because of the decreased LDL receptor activity, and this would also result in more shunting of the VLDL remnants on to LDL. Therefore, alterations in lipoprotein levels in subjects with an E2 or E4 phenotype can be explained by the modulation of these metabolic pathways by apoE.

Important unresolved questions still remain, though. Chief among these is the role of apoE modulated processes in regulating the synthesis and secretion of lipids and lipoproteins by the liver. Regulation at this level by apoE may also be responsible for a portion of the alterations in the lipoprotein concentratons in individuals with the different apoE phenotypes. Much has already been learned about the regulation of lipoprotein metabolic pathways by apoE, but further studies will certainly shed additional important new insights into the role of apolipoproteins and lipoprotein receptors in regulating the metabolism of apolipoprotein B containing lipoproteins.

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DR. SCANU: Dr. Zech, how do you decide on which model to choose? What is the selection criteria?

Dr. Zech: I tried to satisfy as many constraints as I know are on the system. There are not only constraints that have to do with the data that you collect when you do the study, but there are qualitative constraints. There are lots of facts that you know about the system that you can't put into numbers. We try to satisfy all of the mass constraints that are being measured as well as the fact that the tracer is flowing through the system and in some cases where we don't have tracers we just try to look at the mass constraints. We try to put the system into some kind of a state so that we know that the dynamics is not affecting the kinetic study that we want to do and, if we can't do that, we try to examine how the dynamics of the situation is affecting the kinetics.

For example, if you want to study something that turns over fairly fast in the VLDL and you know that the VLDL is changing from one meal to the next because after people eat the VLDL tends to go up or the triglyceride-rich particles tend to go up, you might want to feed the patient every four hours or every six hours and take a sample just before you feed them, if you need points that close together.

Now, that's not changing the fact that things are moving, but you are always taking the sample with respect to the same level of material, if it comes back to that and we've done investigations to look into those kinds of things. What happens is it doesn't change the average answer much, but it changes the fact that you need a lot less patients to get the average answer because the noise goes way down in the study. So, we try to do all of those things. This procedure has been used for a long time before I was doing it. Mones Berman tried to work out a lot of these models. So, it's not something that I want to take credit for, but that's what we try to do. And we don't think that we're describing the system; what we think we're describing is the way we think the system works and we hope that people will try to either prove it or disprove it. In other words, we are not doing confirmatory data analysis that helps build us some hypothesis that can be tested.

DR. GINSBERG: I'd like to follow that with a more specific example that might help people understand some of the processes. In the chylomicron study that you showed, the K curve certainly for triglyceride looked for about 98 percent to be monoexponential. You have about a five or six pool cascade there. Did you get a better fit with the cascade or the same fit or a slightly worse fit and you used the cascade?

DR. ZECH: We started out with a very simple model. We found that model worked on some of the studies but it didn't work on all of the studies. We tried to build a model that would work for all of the studies, but still be as simple as possible. The procedure that helped us the most was to extend it into a cascade. The other procedure we tried was not to have any of the particles being taken out until they got down to be remnants and that just didn't work. Particles needed to be coming out all along the pathway to fit the curve. We built in some feedback going back to the liver, but, as I stated, I'm not sure that's exactly the way that it works. We have one sample in the liver and the data fits that sample, but we don't know whether to approach that from the bottom or from the top. In other words, it is going down in the liver or is it still going up in the liver? We don't even know that it is inside of the liver. All we know is that it is associated with it. So, there are lots of

questions. Not only that, but those were chylomicrons that were taken out of one rat and injected into another. The rat was in a steady state. So, we only see those chylomicrons move through the system. We are not studying the actual system of the donor rat. We are setting this up to just study the effect of lipase on those particular particles.

DR. FISHER: I think these last two papers are particularly exciting. It has been evident to many of us for a long time that the methodology of analyzing kinetic data was more advanced than the experimental sophistication of those of us who were designing the experiments and generating the data. Dr. Witztum and Dr. Gregg have begun to move the types of studies that we are going to have to do in the future in our kinetic investigations to a much more sophisticated level. I commend both of these gentlemen.

DR. GINSBERG: Dr. Gregg, I think that to compare the plasma residence times of E2, E3, and E4 that you would need to have homogeneous labeling of all of the lipoproteins since you are looking at whole plasma removal, yet you showed us very different distributions of the E isoforms and Dr. Weisgraber yesterday gave us a good reason for the differences in distributions. My question is, if E2 and E3 are more bound in a non-exchangeable way to A2 and HDL, could not the more rapid removal of E4 that you see simply be a reflection of VLDL remnant removal with E as a simple passenger on the VLDL rather than any direct effect of E4?

DR. GREGG: We've looked at a number of different things. We've looked at VLDL specifically and shown that E4 is catabolized more rapidly than E3 onto the LDL. We've also looked at the catabolism in each one of the subfractions. In addition, we've chemically modified the ApoE in such a way that we either block the free sulfhydryls to inhibit the ability to form complexes or we block the sulfhydryls in such a way that we change the charge. It is not just the blocking of the sulfhydryls and the inability to form EA2 complexes that lead to the altered catabolism.

SPEAKER: What is it then?

DR. GREGG: Well, to lead to the altered catabolism, you must have both a blocked sulfhydryl plus a positive charge there. It is not just a blocked sulfhydryl that will do it.

DR. GINSBERG: Do you take away the differential distribution of the Es if you block them?

DR. GREGG: Somewhat, but not entirely by just blocking the sulfhydryls. What we do is we block it with a methyl group or an amino ethyl group. If you put on a methyl group it will metabolize more like an E3, but you have to put on the amino ethyl group and put a positive charge on there to really get E4 metabolism out of an E2.

DR. MELCHIOR: A comment to Dr. Gregg and to Dr. Witztum as well. Both of you expressed your turnover data in terms of percent of injected dose cleared per unit time and both of you made statements about catabolic rates. There is a danger, I think it is important to realize that just because the label disappears from the plasma faster in some instances than others that doesn't necessarily mean that the catabolic rate gets faster. In other words, you've removed the effect of pool size on the situation. You, in fact, said that you quantified ApoB in some cases by immunodiffusion, but always expressed the data in terms of fraction of dose remaining. If you don't express it as specific activity, you really can't say anything about catabolic rate, can you?

DR. GREGG: These were in steady state. We measured ApoB all the way along. So the ApoB was in steady state and therefore it is the same number because I only showed one curve. In one case I'm injecting, the E2 LDL into an E3 subject. There is no E2 LDL pool. So, in that case, it really doesn't make any sense to classify or to show it a specific activity. I just wanted to show that these two LDLs are metabolized at a different rate. But, if you look at the E2 LDL in the E2 subject--I was just taking out the whole plasma catabolism--and the ApoB was in constant levels during that time. Therefore, the specific activity decay curve and the fraction of injected dose decay curve are exactly the same.

DR. FISHER: Let's address that question again because I think that's an important one and it comes up time and time again. In utilizing the method of analysis which Dr. Gregg uses and also the one which Dr. Witztum uses, one may present the data either as specific activity or as total dose initially, but in actually analyzing the data it is more convenient to multiply by the pool size in order to model the data as total dose and to keep the pool size, the mass, as a constraint on the model, one then uses that mass and insists that the model fit the mass.

So, whether you present the data as percent injected dose or specific activity, you are still dealing with the same data and the model is dealing with it with mass included.

DR. WITZTUM: I'd just like to make the point that in studies we did we don't know that what we were tracing is a tracing of anything existing because we are not dealing with modal densities. The data was modeled as tracing so that, for example, 100,00 counts were injected and we're saying so many counts disappeared in plasma. The mass in that case is 100,00 counts and the data in terms of pool size were expressed in that. I think for these first approximations and for the purpose of what we were doing pool size is not involved. On the other hand, we can't take the data that we applied and apply it now to state that this patient had this much conversion of this or that. It's just following the tracer.

DR. HAVEL: Dr. Gregg, for your last set of studies, could you tell us more about the properties of the particles containing B-48 and B-100? From this E-deficient patient.

DR GREGG: I showed the lipid composition and the protein composition.

DR. HAVEL: Does the particle size differ between the B-48 particles and the B-100 particles?

DR. GREGG: We have not done a non-denaturing gel or subfractionations or fractionated those across a column. So, I can't give you the exact particles.

DR. HAVEL: In our studies, we felt that--they were one of the influences on the differences between particles. So, I think that's a point for further consideration. The other question I have about the very last studies. You made the point in your characterization of patients or the index patients in earlier studies that there is a lot of B-48 in LDL and now you've injected B-48 back into the patient. Can you tell us more than you told us about this metabolism in that regard?

DR. GREGG: Well, there is some conversion of the B-48 particles into LDL, but it is a relatively small amount of conversion. But, once they get there, they are catabolized at an extremely slow rate. So, because these particles are catabolized so slowly, they stay around in the circulation for a longer period of time. So, if we look at the distribution of B-48 to B-100, say the ratio from VLDL to IDL to LDL, we see that there is a marked difference. There is a large amount of B-48 in VLDL and a moderate amount of B-48 in IDL and there is some B-48 in LDL, definitely some B-48 in LDL, but there is predominately B-100.

If we then take the LDL and subfractionate it by ultracentrifugation and look at a 1019 to 1030, 1030 to 1050 and a 1050 to 1063, again we see that by the time you get down to the 1030/1050 or the 1050/1063 that there is virtually no B-48 there.

DR. KRAUSS: This is by way of clarification of your point that ApoE is important or necessary in some way for the formation of LDL and this is in regards to the ApoE-deficient VLDL. Did you describe the conversion of that VLDL through IDL to LDL? It is impaired in any way?

DR. GREGG: We did not do a paired VLDL/LDL study in this study that I was doing. On the last study we injected VLDL into that ApoE-deficient subject and there was very little conversion of B-100 or B-48 onto IDL and LDL. It really got hung up in the VLDL density range and, if one looks at it, this also goes into the IDL density range. So, even though we did not do formal kinetic analysis of that, there is a marked inhibition of the conversion of specifically B-100 into IDL and LDL. And, if you looked at he synthetic rate of LDL ApoB in that individual, the synthetic rate was I believe two milligrams per deciliter a day. So there was a marked conversion and a marked synthetic inhibition there.

SESSION VI. Dietary Effects on Heterogeneity of ApoB-Containing Lipoproteins

Chair: Scott Grundy

LIPOPROTEIN HETEROGENEITY PRODUCED BY VARIOUS DIETS IN MAN

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Introduction. Several physical techniques, including analytical ultracentrifugation, several varieties of density gradient ultracentrifugation, gradient gel electrophoresis, and gel permeation chromatography, have revealed that very low density lipoproteins (VLDL) and low density lipoproteins (LDL) each are composed of multiple populations. The heterogeneity or polydispersity of LDL is particularly evident in patients with hypertriglyceridemia (type IIb, IV) (1). Normal subjects and patients with isolated hypercholesterolemia (type IIa) tend to have single dominant LDL subpopulations, and only very small amounts if any, of other populations of LDL, i.e. their LDL is paucidisperse. Fisher et al have suggested that LDL heterogeneities are inherited (2).

When some of the major nutrients of the human diet are varied changes result in plasma lipid and lipoprotein concentrations. As a result of diet-induced alterations of lipoprotein production, intravascular processing, or cellular removal, there also may be changes in the sizes, densities, and lipid and apoprotein compositions of the lipoprotein particles themselves. Several dietary variations have been extensively studied which include 1) isocaloric substitutions of dietary triglycerides containing omega-6 or omega-3 polyunsaturated acids for the usual relatively saturated fatty acid-containing triglycerides, 2) increasing the carbohydrate content of the diet at the expense of fats or vice versa, 3) altering the amount of cholesterol, and 4) caloric restriction with or without alteration of the overall composition of the diet. In man, changing the nature of the protein ingested by itself does not appear to have major effects on lipoprotein composition or distribution.

Polyunsaturated fatty acids. Isocaloric substitution of polyunsaturated fatty acids for saturated fatty acids tends to lower the levels of all major classes of lipoproteins, including VLDL, LDL, and high density lipoproteins (HDL) (3,4). The reductions in VLDL and LDL may be due to upregulation of hepatic LDL (apoB,E) receptors directly, because upregulation results in more efficient removal of the apoB- and E-containing lipoproteins from plasma (5). Equimolar amounts of long-chain polyunsaturated fatty acids are less efficient in stimulating VLDL production in the liver than are saturated fatty acids of equivalent chain lengths (6). Polyunsaturated fatty acids may also slow the production of HDL by mechanisms that are not clear (7).

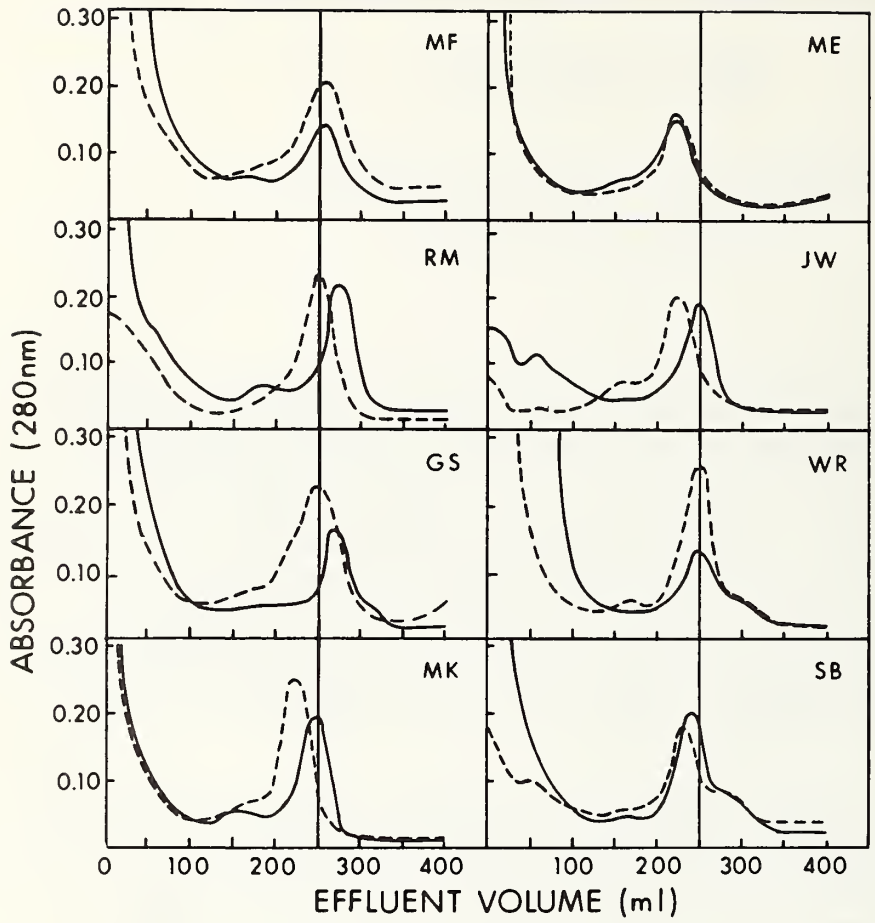

Figure 1. Changes in LDL flotation properties on zonal ultracentrifugation in hypertriglyceridemic subjects in response to weight loss. Plasma triglyceride concentrations of hypertriglyceridemic subjects were reduced by caloric restriction. Lipoproteins were separated by zonal ultracentrifugation: VLDL eluted between 0 and 100 mL and LDL eluted between 200 and 300 mL. Solid lines = pre-weight loss period; dashed lines = post-weight loss period.

* * *

In addition to decreases in concentration of the lipoproteins in response to ingestion of polyunsaturated fatty acids there also are changes in lipoprotein compositions: the fatty acids of lipoprotein phospholipids and cholesteryl esters come to reflect the dietary fatty acids, altering the physical properties of the core and surface regions of the lipoproteins (8,9). Changes in the physical properties of the lipids, in particular changes that would alter fluidity at body temperature, could alter the conformations of apoproteins and thus affect the interactions of lipoproteins with enzymes, transfer proteins and cellular receptors (10). Thus a change from dietary saturated fatty acids to polyunsaturated fatty acids can affect VLDL production, LDL structure, and clearance of lipoproteins mediated by the hepatic apoB,E cellular receptors.

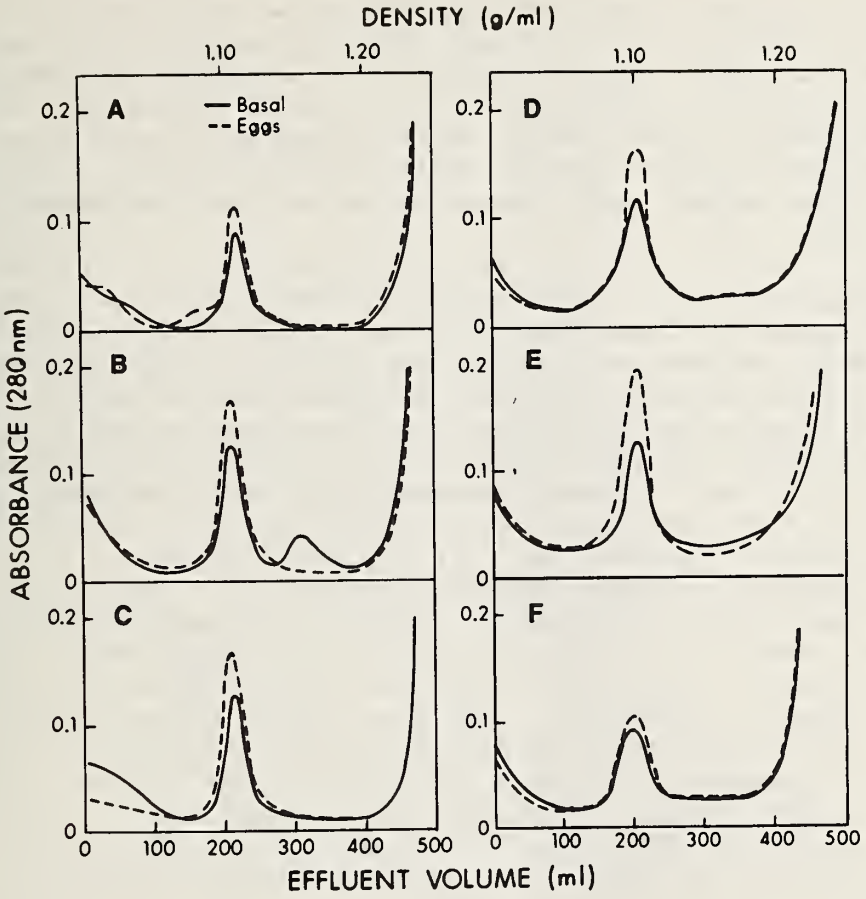

Figure 2. Effects of high-fat, high cholesterol diet on flotation properties of LDL on zonal ultracentrifugation. Normolipidemic subjects were fed diets containing either 30% fat (P/S=0.4) and 250 mg cholesterol/day or 39% fat (P/S=0.4) and 1760 mg cholesterol/day. Plasma lipoproteins were separated by zonal ultracentrifugation: LDL eluted between 170 and 260 mL. Solid lines = low-fat, low-cholesterol diet; dashed lines = high-fat, high-cholesterol diets.

* * *

Omega-3 fatty acids are particularly effective in lowering plasma VLDL levels. In large part, this may be due to the shunting of the fatty acids into oxidative pathways in the liver and away from pathways of esterification, resulting in reduced secretion of VLDL by the liver (11). It is not yet clear whether the particular effectiveness of the omega-3 fatty acids is due to the degree of polyunsaturation or to the omega-3 placement of one of the double bonds, because direct comparisons between omega-3 and omega-6 fatty acids of equal chain length and degree of saturation have not been performed.

The relationships between alterations of dietary fatty acid composition and changes in various specific subpopulations of LDL is

not clear, since most studies have been carried out in subjects with either normal lipid values or isolated hypercholesterolemia, who probably have paucidisperse LDL (1). Even if subjects with polydisperse LDL had been studied, possible effects on LDL subpopulations could have been missed because the LDL have not been studied by techniques most likely to detect changes in LDL dispersity, such as gradient gel electrophoresis or analytical ultracentrifugation. Nevertheless the data suggest that changes in dietary fatty acids do alter LDL structures.

Caloric Restriction. Diet studies have been carried out in hypertriglyceridemic patients who probably have polydisperse LDL. In such patients isocaloric substitutions of omega-6 fatty acids was not studied because such measures do not lower triglycerides very efficiently. Instead, studies were performed that lower triglyceride levels by dietary caloric restriction (12), by use of the medication bezafibrate (13) or by the addition of omega-3 fatty acids (14-16). On the first two treatment regimens LDL subpopulations were characterized before and after treatment by zonal ultracentrifugation. Although the ultracentrifugal profiles indicated that polydispersity remained after lowering of triglycerides, the major LDL population had shifted from a smaller, more dense particle to a larger, less dense particle (Figure 1). The latter contained less protein and triglycerides, and more cholesterol. The major LDL particles after treatment with bezafibrate, demonstrated increased abilities to interact with LDL receptors of cultured cells and altered interactions with monoclonal antibodies suggesting that therapy had modulated the conformation of apoB on the surfaces of the LDL particles. Lipoprotein heterogeneity has not been examined in response to omega-3 fatty acid supplementations.

Treatment of hypercholesterolemic patients with bile acid binding resins alter the compositions and size distributions of LDL particles, but in a manner opposite to that seen in hypertriglyceridemic patients treated with caloric restriction or bezafibrate. Following resin therapy, LDL particles on the average are smaller, triglyceride enriched, cholesterol depleted, and cleared from plasma at slower rates than large particles (17,18). Thus, although heredity may play an important role in determining the degree of polydispersity of LDL in a given person, the nature of the dominant LDL subpopulations also is affected by environmental factors such as diets and drugs.

High Fat, High Cholesterol Diets. Studies also have been carried out to evaluate the responses of normolipidemic and hypercholesterolemic individuals to high-fat and high-cholesterol diets. Most such individuals have paucidisperse LDLs on zonal or analytic ultracentrifugation and respond to increases in total dietary triglyceride-fat, or to increases of both dietary fat and cholesterol, by raising the concentrations of LDL in their plasmas (19-21). However, the LDL particles appear to be unaltered in their behaviors on zonal or analytic ultracentrifugation or on gel permeation chromatography (Figure 2). Nor are significant changes seen in their chemical compositions or interactions with cells. The VLDL ($d<1.006$ g/ml) on

the other hand, acquire subpopulations of particles that are apoE enriched and that react with peritoneal macrophages in an enhanced fashion (22). Subpopulations of HDL also appear that interact with cultured fibroblasts with greater affinity (23). These highly cell reactive species of lipoproteins are present only in small amounts in the plasmas of fasting normal persons, even after several weeks of feeding of high-fat, high-cholesterol diets. This is in contrast to the situation in several species of non-human primates, rats (24), and rabbits where similar diets cause the cell-reactive lipoproteins to accumulate in great quantities in plasma. In normal and hypercholesterolemic man, the most startling and reproducible changes induced by high-fat, high-cholesterol feeding are seen in HDL. HDL_2 concentrations increase significantly and consistently and the proportion of cholesteryl ester in the HDL_2 particles also rises (20) (Figure 3). The responses of the lipoproteins of hypertriglyceridemic subjects to such diets are not known.

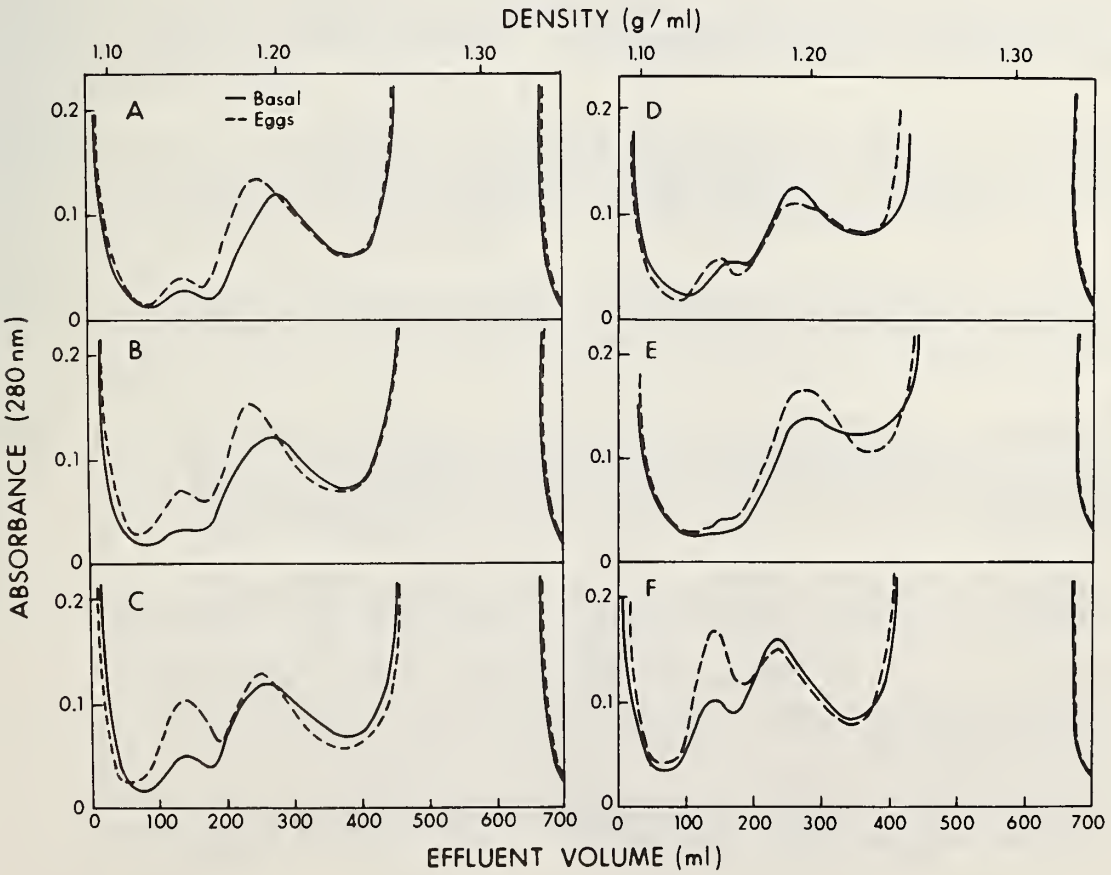

Figure 3. Effects of high-fat, high-cholesterol diet on flotation properties of HDL on zonal ultracentrifugation. Normolipidemic subjects were fed the diets described in Figure 2. HDL_2 eluted between 70 and 180 mL; HDL_3 eluted between 70 and 350 mL. Solid lines = low-fat, low-cholesterol diet; dashed lines = high-fat, high-cholesterol diet.

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HETEROGENEITY OF LOW DENSITY LIPOPROTEINS IN ATHEROSCLEROSIS: NONHUMAN PRIMATE SPECIES AND DIET COMPARISONS

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Introduction

Low density lipoproteins, or LDL, are defined as lipid-protein complexes, or particles, that are isolated from blood plasma within the density range of 1.019 to 1.063 g/ml. ApoB-100 is the major protein and cholesteryl esters make up about 50% of the lipid. The particles are spherical with cholesteryl esters in the center and protein and phospholipid on the surface of the sphere. Although this definition is accurate and reasonably specific, it is ambiguous and a semantic problem in defining LDL remains. This is because of several factors. The LDL of a single individual exist as discrete subpopulations of particles of several different compositions and the rules governing the number and types of subpopulations within any one individual are unknown. The LDL of different individuals exist as populations of particles with distinguishable characteristics. The LDL of different individuals respond to dietary cholesterol and fat with detectable composition changes. The LDL of different species may respond to diet with characteristic differences. Due to these uncertainties, the term LDL heterogeneity is frequently used.

In nonhuman primate models for the study of atherosclerosis, significant diet-induced modification of LDL occurs depending on the species (1-3). One measure of diet-induced modification, the agarose column chromatography-determined average molecular weight, is more highly correlated to the extent of coronary artery atherosclerosis than most other lipoprotein and cholesterol measurements (3-5). This finding highlights the need for understanding LDL heterogeneity, as do the suggestions of others that selected LDL subpopulations are predictive of the degree of coronary heart disease in man (6,7). The goal of this presentation is to describe the types of changes that occur in LDL upon dietary induction of atherosclerosis in monkeys, and to contrast the responses in two different species. The extent of modification is greater than occurs in humans, but the underlying reasons for the changes may not be so different, as discussed previously (8). In any case, determination of the mechanisms that lead to the patterns of LDL heterogeneity seen in monkeys will generally enhance our understanding of LDL formation.

Species Comparisons of Diet-Induced Atherosclerosis

In general, addition of cholesterol and saturated fat to the diet is sufficient to induce hypercholesterolemia in monkeys if fed for a period of 1.5 to 5 years. The data in Table 1 contrast the plasma cholesterol and lipoprotein concentration changes that occur in two different species of monkeys with markedly different sensitivity to diet induced atherosclerosis. The African green monkey makes a modest dietary response relative to the cynomolgus monkey. In both species, the cholesterol concentrations of total plasma, LDL, and VLDL plus ILDL are significantly higher in the group fed the higher cholesterol level. The mean HDL cholesterol concentration was significantly lower in the high cholesterol-fed cynomolgus monkeys and although the mean value for HDL cholesterol was not different in the two groups of African green monkeys, the higher responding green monkeys also had lower HDL cholesterol concentrations (9). The average LDL molecular weight is significantly higher in the high cholesterol groups of both species, and this difference is greater in cynomolgus monkeys.

Table 1. Dietary Cholesterol Effects on Plasma Lipoproteins in Monkeys.

Species	Diet Chol.	n	Whole Plasma	V+ILDL	LDL	HDL	LDL MW
			cholesterol concentration (mg/dl)				(g/umole)
Cyno-molgus	Low	8	140 12	12 2	73 12	55 8	3.2 0.2
Cyno-molgus	High	7	648* ±84	83* ±15	541* ±68	21* ±3	5.5* ±0.3
African Green	Low	8	150 ±10	7 ± 1	56 ± 4	81 ± 4	3.2 ±0.1
African Green	High	11	277* ±37	26* ± 7	153* ±18	97 ±15	3.7* ±0.1

All values, mean ± SEM. All diets contained 40% of calories as saturated fat. Low cholesterol diets contained 0.15 or <0.1 mg of cholesterol/kcal and high cholesterol diets contained 0.8 or 1.0 mg/kcal, for African green and cynomolgus monkeys, respectively. Lipoprotein cholesterol concentrations and LDL molecular weights were determined after lipoproteins were separated by agarose column chromatography (11).

* Significantly different from Low Chol., $p<0.01$.

The data in Table 2 illustrate the difference in the coronary artery intimal area as a measure of atherosclerosis severity in the two diet groups for each species. Significantly higher values were obtained in the

groups fed more dietary cholesterol for both species. While these numbers serve to illustrate the coronary artery atherosclerosis response to diet, they do not show the rather major difference in the quality of diet-induced atherosclerosis in these two different species (see Figure 1). In addition to the greater amount of intimal area, the cynomolgus monkeys have concentric lesions and ectasia that appears to maintain the same lumen diameter in spite of developing atherosclerosis. Therefore, the type as well as the amount of coronary artery atherosclerosis appears different between these two species and this is a factor to be kept in mind when comparisons of atherogenic properties of lipoproteins between species are made.

Table 2. Diet Induced Coronary Artery Atherosclerosis

Species	Diet Chol.	n	Intimal Area (mm^2)
Cynomolgus	Low	5	0.02 ±0.10
Cynomolgus	High	7	1.57* ±0.11
African green	Low	4	0.02 ±0.01
African green	High	10	0.30* ±0.19

All values, mean ± SEM. Diets were as in Table 1, and were fed for thirty months and five years for cynomolgus and African green monkeys, respectively. Intimal area was measured morphometrically in 15 sections including all coronary arteries (4).
*Significantly different from Low Chol., $p<0.05$.

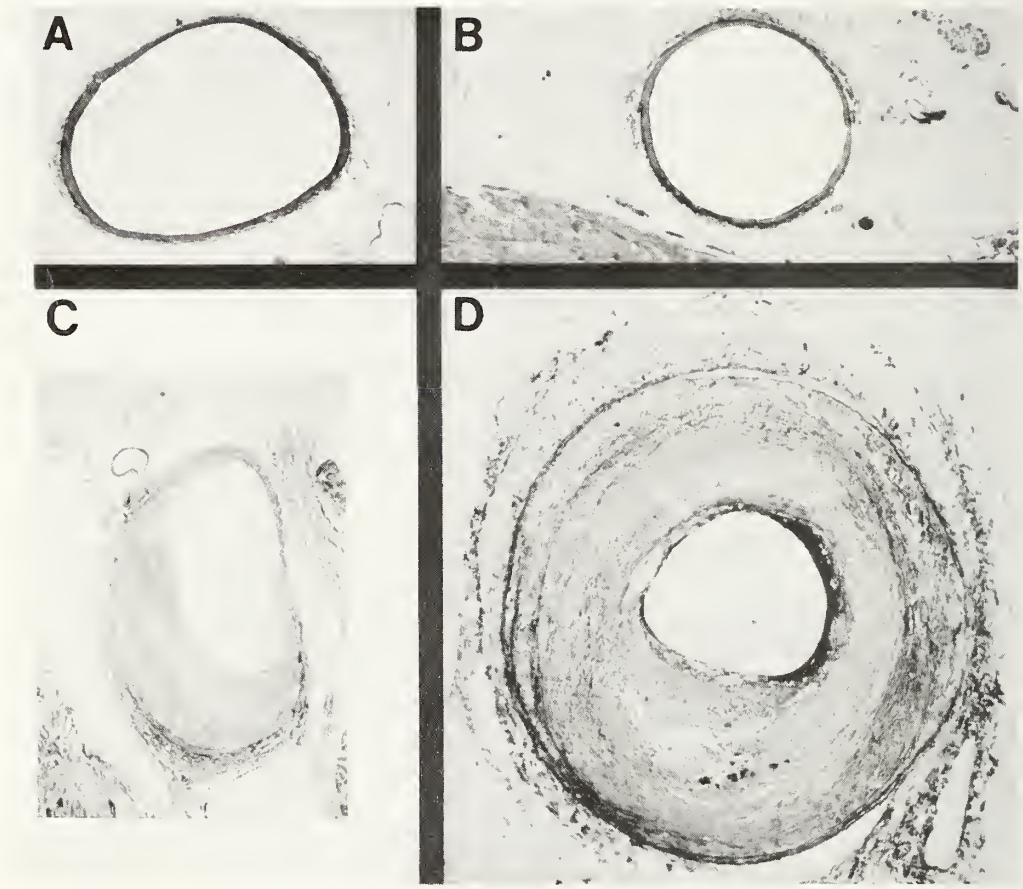

Figure 1. Representative sections of coronary arteries from male cynomolgus and African green monkeys from the experiments described in Table 2. All hearts were perfusion fixed at a pressure of 100 mm Hg and all sections have been printed at the same magnification. **A.** Coronary artery from an African green monkey fed a low cholesterol diet for 5 years. **B.** Coronary artery from a low cholesterol diet fed cynomolgus monkey fed for 30 months. **C.** Coronary artery from an African green monkey fed a high cholesterol diet for 5 years. A significant eccentric lesion is present that contains foam cells, fibrosis, and extracellular debris near the base of the lesion. In this case, lumen diameter has been narrowed. **D.** Coronary artery from a cynomolgus monkey fed a high cholesterol diet for 30 months. Significant atherosclerosis is present with foam cells, fibrosis, calcificaton, and sterol clefts being apparent. The lesion is concentric and it should be noted that lumen area has been maintained so that it remains similar to that of the low cholesterol diet cynomolgus monkey.

Another factor to be considered when evaluating atherogenic properties of lipoproteins is the responsiveness of individual arteries. We have shown, using African green monkeys, that several lipoprotein endpoints are highly correlated with the severity of atherosclerosis, measured morphometrically as intimal area in these different arteries (4). However, as can be seen in Table 3, multiple regression analysis showed that the average size of LDL was the property most predictive of the severity of coronary artery atherosclerosis in these animals, while LDL particle concentration was more important in predicting severity of abdominal aorta and carotid artery atherosclerosis. This outcome suggests that different properties of LDL may be relatively more atherogenic depending on tissue specific factors yet to be identified. It is significant to note that in all three arteries, one of the properties of LDL was selected by multiple regression analysis from several lipoprotein measurements to be the most predictive of atherosclerosis severity. In addition, the data show that from 70 to 90% of the variability in atherosclerosis severity was explained by the lipoprotein measurements.

Table 3. Multiple Regression Analysis of Abdominal Aorta, Coronary Artery and Carotid Artery Atherosclerosis vs Plasma Cholesterol and Lipoprotein Measurements

	R^2	Significance (of the regression)
Abdominal Aorta Intimal Area with:		
LDL particle conc. (uM)	0.83	p<0.0001
LDL MW (g/umole)	0.87	
HDL mass (mg/dl)	0.89	
Total plasma chol (mg/dl)	0.89	
CE ratio (CO/CL)	0.89	
Coronary Artery Intimal Area with:		
LDL MW (g/umole)	0.64	p<0.0001
HDL mass (mg/dl)	0.68	
Total plasma chol (mg/dl)	0.70	
LDL particle conc. (uM)	0.71	
CE ratio (CO/CL)	0.71	
Carotid Artery Intimal Area with:		
LDL particle conc. (uM)	0.65	p<0.0001
Total plasma chol (mg/dl)	0.75	
CE ratio (CO/CL)	0.77	
LDL MW (g/umole)	0.78	
HDL mass (mg/dl)	0.78	

Atherosclerosis was quantitated morphometrically in African green monkeys fed an atherogenic diet for 25 months and plasma lipoprotein measurements were made as described (4).

Species Differences in LDL Particle Characteristics

Because cynomolgus and African green monkeys respond so differently to the same level of dietary cholesterol, similarities in the pattern of response in plasma lipoprotein metabolism may be obscured. Therefore, we carried out a study in which groups of each species were fed different dietary cholesterol levels to obtain comparable degrees of hypercholesterolemia (10). As shown in Table 4, the LDL cholesterol concentration was similar for both groups, but the average LDL molecular weight was higher in the cynomolgus monkeys. For analysis, the LDL from these animals was then isolated using agarose column chromatography (11) and these LDL were subfractionated by density gradient ultracentrifugation using a vertical rotor (10). The profile for LDL isolated from a single cynomolgus monkey is shown in Figure 2B. Clearly the material isolated in the LDL region from the column is heterogeneous and contains several density subpopulations, as indicated by the peaks and shoulders. After this separation, the material from individual tubes was then analyzed by electrophoresis in a 2 to 8% polyacrylamide gradient gel to determine if homogeneous size populations could be identified. The gel photograph of Figure 2A shows that even for the material in individual tubes from the density gradient, multiple bands could usually be detected. The typical pattern for any tube was for a main band and one or two minor bands to be present. When the starting material was run (lane T), it was clear that the LDL were heterogeneous and material was present in all of the regions identified for individual density gradient fractions. However, it was not until the LDL had been fractionated by density gradients that many of the numerous discrete size fractions present in the starting material became apparent. The challenge remaining is to determine the numbers of bands within the LDL fraction of any one animal and the actual pattern among different animals for the different size fractions.

Table 4. Comparison of LDL Between Species with Comparable Hypercholesterolemia

Species	n	TPC	LDL Chol.	LDL MW
		(mg/dl)		(g/umole)
Cyno-molgus	6	430 ± 139	366 ± 130	4.1 ± 0.5
African Green	5	424 ± 181	345 ± 168	3.5* ± 0.4

All values, mean ± SD. Data from reference 10.
*Significantly different from Cyno, $p<0.05$.

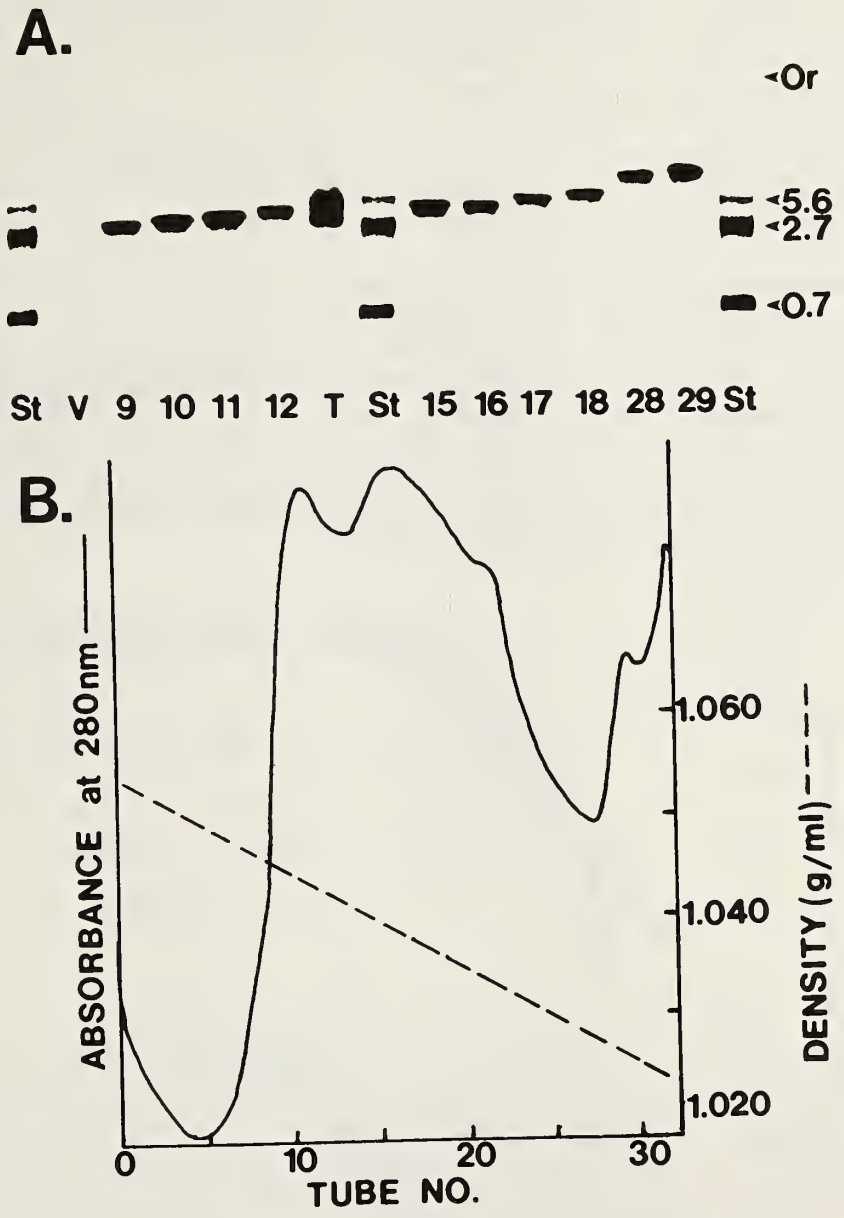

Figure 2. **A.** Separation by 2-8% polyacrylamide gradient gel electrophoresis of density gradient fractions isolated as in B. Fractions are numbered by tube number as shown in B. St marks standard lane, V marks VLDL, and T marks total starting LDL. Molecular weights of standards are shown on the right (in millions) and Or marks the well of the horizontal gel. **B.** Elution profile of an LDL sample from a single cynomolgus monkey separated by centrifugation in a vertical rotor as described before (10).

This type of size and density heterogeneity was seen in both cynomolgus and African green monkey LDL, although it appeared to be more pronounced in LDL of the cynomolgus monkeys (10). Perhaps this could be related to the increased size responsiveness of the LDL of this species to dietary cholesterol, although at present this is only speculation. Further analyses were done in an attempt to compare characteristics of LDL between the two species. The data in Figure 3 show cumulative distribution patterns of cholesterol among LDL fractions in the density gradients for both groups of African green and cynomolgus monkeys (10). For the cynomolgus monkey LDL, cholesterol isolated in lower density regions of the gradient than for African green monkey LDL. This finding shows that the LDL subfractions of the cynomolgus monkeys are consistently less dense than those of African green monkeys. However, when the percentage compositions of the various gradient fractions were compared based on density, as shown in Figure 4, the data from both species fit the same lines. This suggests that the particles isolated from the two species are of basically the same spherical structure, but that cynomolgus monkeys have larger, less dense, more cholesteryl ester enriched LDL particles.

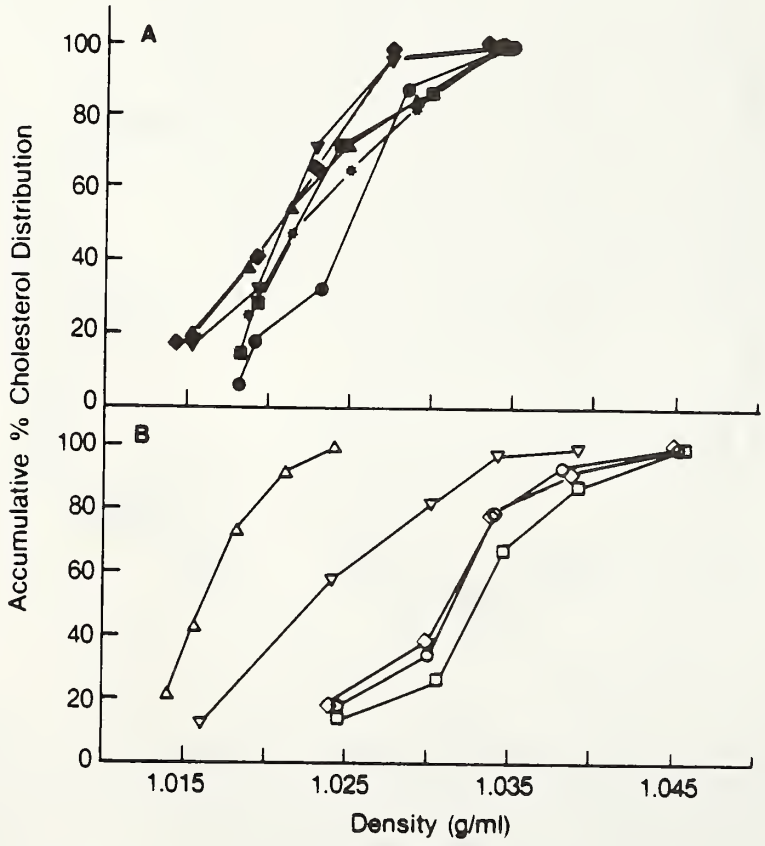

Figure 3. Accumulative percent cholesterol distribution for each cynomolgus monkey (A) and African green monkey (B) LDL sample. The percent of total cholesterol was summed from the least to the most dense subfraction and plotted against mean density. Each symbol represents a different animal. Data from reference 10.

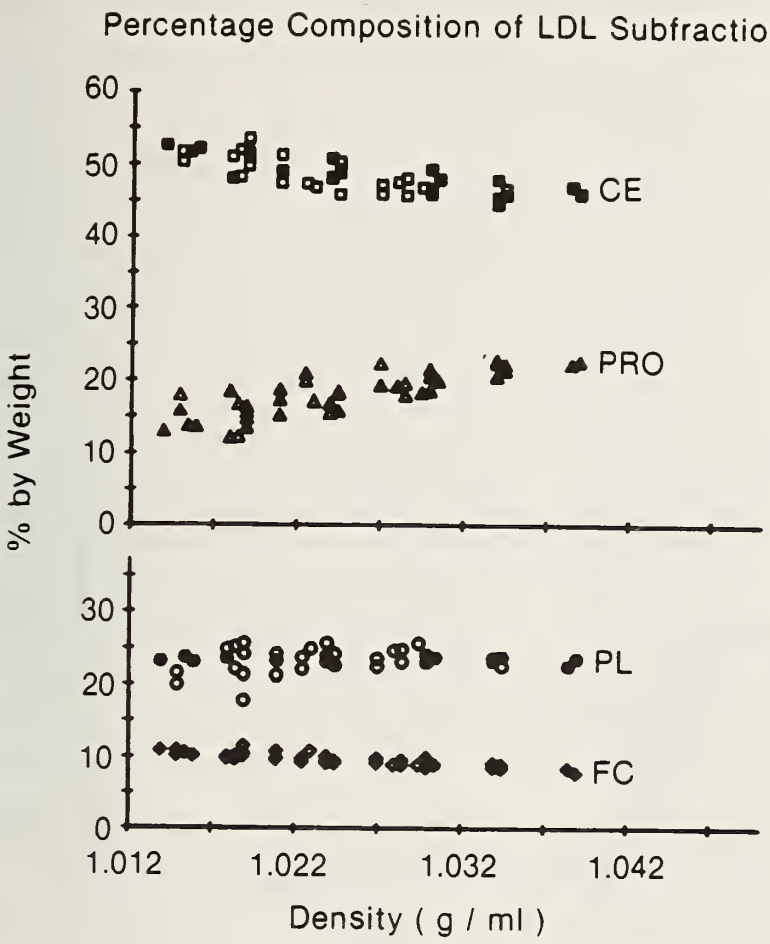

Figure 4. The relationship between mean density and percentage composition of cholesteryl ester (CE), protein (PRO), phospholipid (PL), and free cholesterol (FC) for each LDL subfraction. Open symbols represent LDL subfractions from cynomolgus monkeys and closed symbols represent those of African green monkeys. Triglyceride averaged less than 1% throughout and did not change with density. Data from reference 10.

The apoE, apoA-I and apoB content of the various density subfractions of LDL were also compared by doing ELISA for each apoprotein (12) on individual tubes collected from the gradient eluate. Figure 5 shows the apoE/apoB and apoA-I/apoB ratios for African green monkey LDL. The apoE/apoB ratio was consistently higher in the less dense subfractions of the gradient, even though as in the example, a small proportion of the total mass was present in this region. In general, the cynomolgus monkey LDL subfractions had a higher apoE/apoB ratio than the African green LDL (10), but they also generally were less dense subfractions. Only small amounts of apoA-I were found, however it appeared that a subpopulation of particles with apoA-I (higher apoA-I/apoB ratio) could be identified near the higher density end of the gradient. These may represent HDL_1-like particles and be separate from the apoB containing particles, but further analyses are needed to confirm this possibility. If indeed such HDL_1-like particles were present, it is interesting that they do not apparently contain apoE since the apoE/apoB ratio does not increase near the bottom of the gradient.

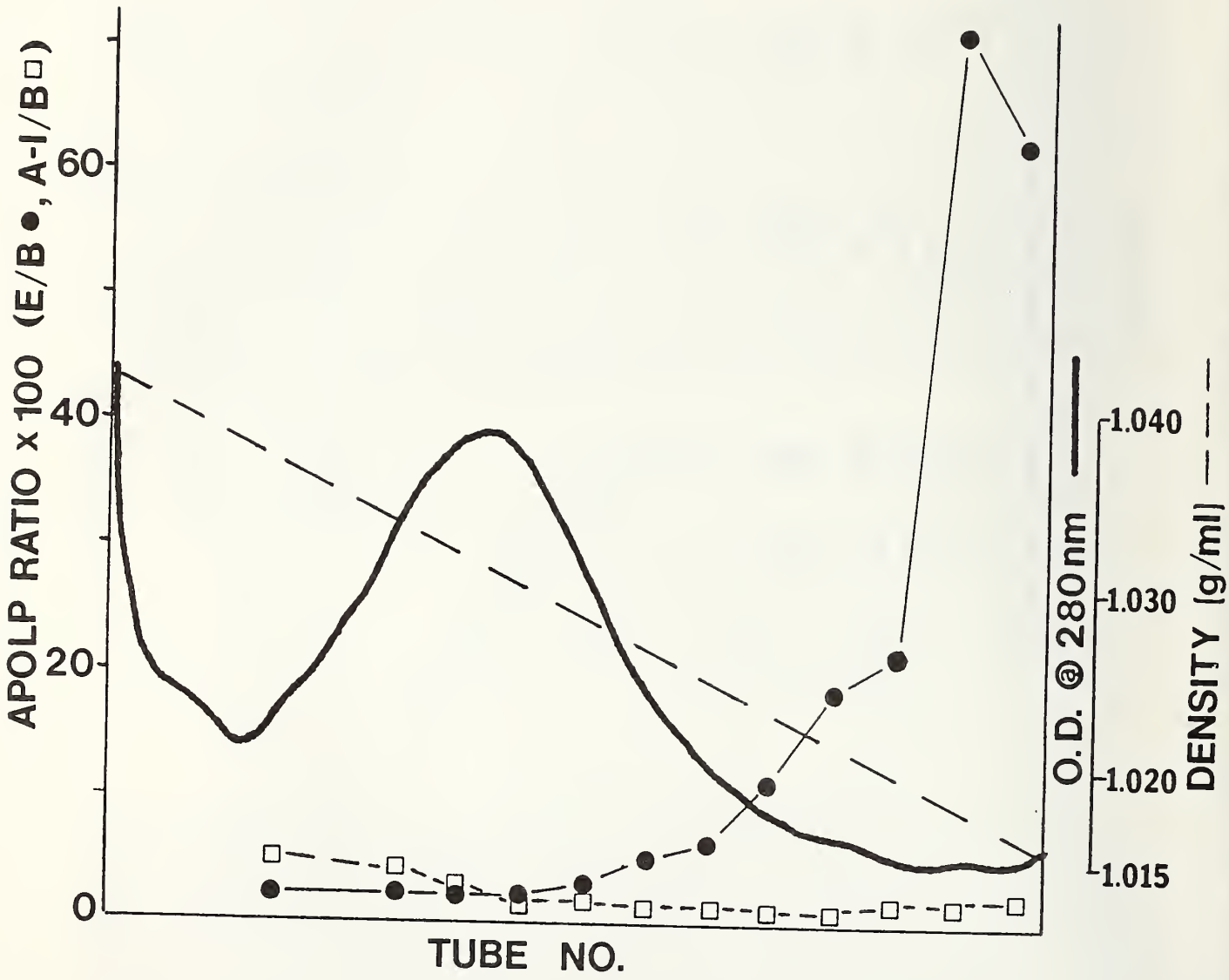

Figure 5. Elution profile of an LDL sample from an African green monkey separated by density gradient centrifugation as in Figure 2. Individual tubes from the gradients of four different animals were assayed for apoA-I, apoB, and apoE by ELISA according to a procedure similar to that described (12), and the mean apolipoprotein (apolp) ratio was plotted. Density was measured by refractometry.

LDL Cholesterol and Cholesteryl Ester Composition

Species and diet differences in LDL cholesterol composition have been further analyzed to determine if characteristic properties could be found that may help explain a relative increase in atherogenicity. In data that we published earlier (13), we saw a proportional increase with particle size in the cholesterol/phospholipid ratio in cynomolgus monkey LDL. These monkeys were all fed saturated fat and cholesterol containing diets and they became very hypercholesterolemic, i.e. TSC=823 ± 41 (mean ± SEM). In current studies, data has been collected from cynomolgus monkeys in which considerably less cholesterol has been fed so that plasma cholesterol concentrations are lower and remain in the range of most humans at increased risk for heart disease due to elevated cholesterol. Comparisons with polyunsaturated fat-enriched diets have also been made as have comparisons of diet effects in African green monkeys and cynomolgus monkeys. Table 5 shows the data for the free cholesterol/phospholipid ratio for the LDL of these groups. There was a positive correlation between LDL size and FC/PL for the saturated groups of each species ($r=0.45$, $p<0.05$), but there was no correlation between this ratio and LDL size in the polyunsaturated fat groups. The average ratio for FC/PL was higher for the polyunsaturated fat fed animals of each species, but differences between dietary fat groups were small and not statistically significant. It does not seem likely that the small differences in the FC/PL ratio observed in these studies will confer a difference in the relative atherogenicity of different LDL in the animals of these studies.

Table 5. Dietary Fat Effects on LDL Free Cholesterol to Phospholipid Ratio in Two Nonhuman Primate Species

Species	Diet	n	TSC (mg/dl)	LDL-CH (mg/dl)	LDL MW (g/umole)	LDL FC/PL (w/w)
Cynomolgus	Sat	14	301 ±28	197 ±31	3.4 ±0.1	0.407 ±0.015
Cynomolgus	Poly	15	291 ±25	214 ±27	3.6 ±0.1	0.434 ±0.015
African green	Sat	15	392 ±32	274 ±38	3.3 ±0.1	0.406 ±0.017
African green	Poly	16	305* ±40	204* ±43	3.3 ±0.1	0.424 ±0.022

All values, mean ± SEM. All diets contained 40% of kcal as fat; saturated fat (Sat) diets have P/S ratio of 0.3 and polyunsaturated (Poly) diets have P/S ratio of 2.2. Diets were fed for at least 6 months before lipoproteins were isolated (11). The cholesterol content was 0.2 mg/kcal and 0.8 mg/kcal for the cynomolgus and African green monkeys, respectively.

*Significantly different from Sat, $p<0.05$.

Another aspect of dietary effects on LDL composition that may be atherogenic is the altered cholesteryl ester composition. Significant changes in the types of cholesteryl esters in LDL particles are induced by high dietary cholesterol levels and different types of dietary fat (8,13). The enlargement of LDL in cholesterol-fed animals has been shown to be accompanied by a selective increase in saturated and monounsaturated cholesteryl esters (13). The data in Table 6 show the modifications in cholesteryl ester content that occurs in LDL from saturated and polyunsaturated fat-fed African green and cynomolgus monkeys. The data are expressed as molecules of cholesteryl ester per LDL particle. The number of cholesteryl linoleate molecules per particle is higher in polyunsaturated vs saturated fat fed animals. This occurred even when the polyunsaturated fat diet resulted in smaller LDL particles in which the total number of cholesteryl esters per particle was less as in the polyunsaturated fat fed African greens. Increased numbers of saturated and monounsaturated fatty acid-containing cholesteryl esters were found in the LDL of the saturated fat-fed animals in both species. However, the data suggest that the number of cholesteryl linoleate molecules per LDL particle is greater in African green than in cynomolgus monkeys when size and diet are taken into account. Additional species differences were the higher cholesteryl arachidonate and lower cholesteryl stearate content of the LDL of African green monkeys. In sum, several differences in cholesteryl ester composition, including those due to size alone, occurred between species that may have significance in determining the degree of atherogenicity of LDL.

The differences seen in cholesteryl ester composition, and the fact that there are low numbers (<100) of triglyceride molecules in these LDL, suggest that the physical state of the core lipids may be different among the groups. The peak melting temperature, T_m, of LDL, the temperature at which the core liquid crystalline-to-liquid transition occurs, was positively correlated with LDL molecular weight ($r=0.65$, $p<0.01$). The T_m was also positively correlated to the CE ratio (monounsaturated plus saturated/polyunsaturated cholesteryl esters) ($r=0.79$, $p<0.01$). A summary of the data is shown in Table 7. The polyunsaturated fat fed animals generally had LDL with T_m below body temperature (39°C) suggesting that these LDL had liquid or melted cores *in vivo*. The T_m of LDL from saturated fat fed animals averaged above 39°C for both species suggesting that these LDL had cores in a liquid crystalline state *in vivo*. This difference in physical state of the LDL may be an atherogenic feature, although the mechanism by which this would occur is still only a matter of speculation. It is hoped that by comparing the amount of atherosclerosis in animals with large, but cholesteryl linoleate-rich LDL, to that in animals with comparably sized LDL with a predominance of more saturated cholesteryl esters, we will be able to determine if size or physical state of LDL is more important in atherogenesis.

Table 6. Dietary Polyunsaturated Vs. Saturated Fat Effects on LDL Cholesteryl Ester Composition in African Green and Cynomolgus Monkeys.

Species and Diet	n	LDL MW (g/umole)	C.E.	20:4	18:2	18:1	16:0	18:0
Cynomolgus								
Sat	12	3.89 ±0.10	2761 ±301	100 ± 2	858 ±26	1192 ± 50	384 ±13	227 ±13
Poly	11	3.89 ±0.20	2757 ±155	64 ± 3	1373* ±74	956* ±65	254* ±23	110* ± 9
African green								
Sat	9	3.70 ±0.19	2595 ±169	221 ± 0	784 ±31	1072 ±111	368 ±13	150 ±22
Poly	10	3.31* ±0.15	2257* ±134	135* ± 7	1218* ±60	604* ±73	245* ± 8	56* ± 5

All values, mean ± SEM. Diets were the same as for Table 5. Cholesteryl esters were separated by HPLC as described previously (14).
Abbreviations: C.E., cholesteryl esters; 20:4, cholesteryl arachidonate; 18:2, cholesteryl linoleate; 18:1, cholesteryl oleate; 16:0, cholesteryl palmitate; 18:0, cholesteryl stearate.
*Significantly different from Sat, $p<0.01$.

Table 7. Dietary Polyunsaturated Vs. Saturated Fat Effects on Physical Properties of LDL in African Green and Cynomolgus Monkeys

Species	Diet	n	LDL MW (g/umole)	T_m* (°C)
Cynomolgus	Sat	12	3.89 ±0.10	43.1 ±0.5
Cynomolgus	Poly	11	3.89 ±0.18	38.8** ±0.3
African green	Sat	8	3.82 ±0.18	39.9 ±0.7
African green	Poly	10	3.31* ±0.15	35.6** ±0.4

All values, mean ± SEM. T_m is the peak melting temperature measured by differential scanning calorimetry. Diets were the same as for Table 5.
*Significantly different from Sat, $p<0.05$.
**Significantly different from Sat, $p<0.001$.

Synopsis of Potential Atherogenic Features of LDL

Several features of LDL have been noted to change in nonhuman primates fed atherogenic diets, suggesting that these changes may define the atherogenic potential of LDL, per se. These features of LDL include: 1) high concentrations, 2) large size with modified cholesteryl ester composition , 3) crystalline cholesteryl ester core, 4) increased particle heterogeneity, and 5) modified apoprotein composition. Many of these changes may cause or result from increased production or decreased catabolism of LDL. Combinations of these or other unidentified properties of LDL could act together to result in increased development of atherosclerosis, but further work will be required to determine the relative importance of each.

Acknowledgements

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SESSION VI. DISCUSSION OF DIETARY EFFECTS ON HETEROGENEITY OF ApoB-CONTAINING LIPOPROTEINS

DR. BARTER: Dr. Schonfeld, about a year ago we published the results of the distribution of HDL particle size as a function of triglycerides and found that in men, as distinct from women, the HDL-2 tends to disappear at quite low triglyceride levels and the HDL-3 becomes an HDL-3B rather than an HDL-3A as judged by gradient gels, again at relatively low triglycerides. We are talking about 150 to 200mg/dl. In your hyperlipidemic subjects who lost weight and decreased their triglyceride level, most of them were still above 150. That's a level at which I wouldn't have predicted a significant change in the HDL particle size distribution or density distribution whereas in the normals, some of them came from up around 150 or 200mg/dl where I would have predicted a small amount of HDL2 and a relatively small HDL3. Some of them down to under 50mg/dl where I would have predicted the greater rise in HDL-2. So rather than saying there is a fundamental difference between hyperlipidemics and normals, it's really a function of what the final concentration of the LDL is and that the effects are pronounced at a much lower level than we might have previously thought.

DR. SCHONFELD: What is the mechanism for what you are saying?

DR. BARTER: I believe the mechanism is a combination of lipid transfers and hepatic lipase and I might talk a little bit about that tomorrow morning.

DR. SCHONFELD: I'm still not convinced by what you say for the simple reason that there were a few of those people who we called hypertriglyceridemic based on the history of having hypertriglyceridemia rather than the initial levels because some of those people who we called hypertriglyceridemic, were very close in triglyceride levels to the controls and, when they lost weight, their levels were very close to those of the controls and yet their HDL's were weird looking to begin with and stayed weird looking even after the weight reduction. That may relate to unknown mechanisms in hypertriglyceridemia. For example, I don't know what the transfer protein concentration is in people who have a tendency to hypertriglyceridemia.

DR. BRUNZELL: Dr. Schonfeld the kind of hypertriglyceridemia patients have is important. We've published some data previously looking at HDL in people who were hypertriglyceridemic who had familial hypertriglyceridemia and their A1 and A2 levels were normal, but their buoyant HDL were triglyceride-enriched. If you select somebody who's got familial combined hyperlipidemia, with the same degree of hypertriglyceridemia their A1 levels are down, they're missing a subfraction of buoyant HDL, as Marion Cheung will show tomorrow. So, the type of hypertriglyceridemia is a major determinant of what happens in HDL.

DR. FISHER: I would like to back that up further. When one looks at the profile and the dispersion of LDL in the hypertriglyceridemic, what we call a polydispersed patient, as contrasted to the normolipemic individuals, you can change the dietary perturbations in the normolipemic and it doesn't make a lot of difference on the profile, but, if you take a hypertriglyceridemic and bring them down to a normal diet and then perturb them further, you've got a different profile than you do in the normal for the LDL.

DR. SCHONFELD: Are you contrasting hypertriglyceridemics with polydispersed versus monodispersed on normals versus--

DR. FISHER: I'm talking about polydispersed hypertriglyceridemics.

DR. SCHONFELD: Okay.

DR. NESTEL: A couple of comments. You did show just one slide of Roger Illingworth on the omega-3 fatty acids. In fact, because there is virtually no VLDL with the feeding of omega-3 fatty acids. In the normal, exchange of cholesteryl ester between particles is presumably greatly reduced and one finishes up with HDL that are extremely cholesteryl ester-enriched and very TG-depeleted and also the same applies for LDL. I'm not sure what the implications of that are for normal or abnormal metabolism. Regarding cholesterol feeding, you discussed LDL and HDL, but you didn't discuss VLDL and IDL. We found a number of years ago that VLDL production was decreased, but there was a shift to a very small VLDL particle which was cholesterol-enriched and ApoE-enriched and again that might have implications for disease.

DR. GRUNDY: Dr. Schonfeld, a lot of us are prescribing low fat diets for people and it lowers the HDL cholesterol sometimes, as you showed, but it seemed to me that your A1 levels were not changed. When you go on a low fat diet, high carbohydrate diet, do you think there is an adverse affect on the HDL pattern?

DR. SCHONFELD: No, I don't think so. In most people, if we are to believe what we know about HDL being somewhat responsible for the transport of cholesterol away from the tissues, it probably just reflects the decreased necessity for that taking place as the cholesterol is removed from the diet. So, I don't worry about that. From what one knows about atheroma and the cholesterol content of atheroma, the lipoproteins that circulate are not cholesterol-enriched, chances are better that whatever lipoproteins get into the artery wall will have the triglyceride removed from them and there will be a smaller residue of cholesterol left to build atheroma with. So, I think that low cholesterol diets are alright. High carbohydrate diets are okay too.

DR. WITZTUM: Dr. Rudel, just a point of information. You always categorized these LDL from the cynomologus monkeys, by chromatography. In looking at some of your gradients a lot of the material is actually floating. Is that right?

DR. RUDEL: Yes, that's correct.

DR. WITZTUM: Others talk about IDL being atherogenic. Is this really equivalent of IDL in the monkey?

DR. RUDEL: That's a good question and I don't know the answer. We are trying to work toward defining the characteristics of the particle based on size and apoprotein composition. We've certainly given you percentage composition. The percentage composition slide from Dr. Marzetta's data that showed that all the percentage composition fit on these tie lines and the particles are all similar in that regards, which suggests to me that these populations of particles have some sort of common metabolic origin based on some perturbation, I presume, in the liver and the liver secreting extra cholesterol in these particles and it's remaining in the particle. I don't think that is an analogous process to what we think of IDL. So, we haven't called it IDL. We don't yet have enough information to tell you metabolically that these are or are not the equivalent of IDL in humans. They have some differences, but there's also suggestions of some similarity.

DR. SCANU: Dr. Rudel, that is a nice gel you have there. Our experience has been that the incidence of LP(a) in the rhesus monkey is quite high, up to 40 or 50 percent in concentration. Have you done any western blots to see whether or not all of them are ApoB-100?

Dr. Rudel: We've done a lot of work with that. Actually, when LP(a) is present it shows up in the bottom of that gradient and you can actually pick it up as being LP(a). The LP(a) from every animal is a little bit different in size and it won't line up exactly with that rhesus monkey LP(a) standard that we used on there, but it will be up there at the top and it will be at the bottom of the gradient. So you can see LP(a) when it is present. It is on the dense end of the gradient and stays down there.

DR. KRAUSS: I have a comment and a question. The comment relates to the question that Joe Witztum asked about possible homologies between what looks like a very buoyant LDL and IDL. We have to back away from the assumption that all IDL is somehow an intermediate metabolite and as you pointed out yesterday, Dr. Rudel, some of that material could go primary secretory in some species, perhaps not in others. Whether or not that is the case, we find in humans, as I mentioned yesterday, a cholesterol-enriched form of particle in the traditional IDL density range that, in looking at Dr. Marzetta's data, appear to have quite a bit of homology in terms of size and composition. We've called this a cholesteryl ester IDL-1 and IDL-2. Now, whether or not that comes down to the molecular level or not, I can't say, but it is certainly attractive to think about that as a potential area for homology, whether you consider this an intermediate or a primary secretory product.

DR. RUDEL: I couldn't agree more. I'd like to say that there probably are analogies. The characteristics that we see in the African green monkey livers when we do perfusions is they contain cholesteryl ester and I don't necessarily pretend that that cholesteryl ester is treated without being modified, but the relationship between the liver cholesteryl ester content and the cholesteryl ester enrichment of our precursor lipoproteins that come out of the perfusate is striking. Maybe that's why we see what looks like ACAT-derived esters in saturated fat-fed animals in plasma LDL's and these esters get secreted. There is an enrichment. Fred Johnson has done a lot of work trying to characterize the secretory products in liver perfusions as affected by the two diets, the polyunsaturated and the saturated fat-containing diets. The bottom line for all of that work which I didn't have time to summarize today, is that there seems to be a relationship between hepatic liver cholesteryl ester content and cholesteryl ester enrichment of the ApoB containing lipoproteins in the perfusate. So, I'd love to know in some of these patients that have some of the IDL-1/IDL-2 particles that Dr. Krauss has looked at, what their liver is doing? What is in their liver? Do they have cholesteryl ester accumulation? Is there a difference in cholesteryl ester accumulation among patients and so on and so forth. It's a fascinating question. I just don't know the answer.

DR. KRAUSS: Dr. Rudel, do you have any information on the possibility of apoprotein polymorphisms in E as well as possibly B? This fits with regard to the IDL question.

DR. RUDEL: Not much. Jan Breslow showed several years ago that the ApoE polymorphism or the several years ago that the ApoE polymorphism or the E2, E3, E4 system that we find in humans doesn't occur in monkeys. Monkeys don't seem to have those same amino acid substitutions. So, the E2, E3, E4 question is not going to be approached in monkeys. In terms of the ApoB, if I had to guess I would say there is a lot more polymorphism certainly in the cynomolgus monkeys if that is partly what's related to all of that paucidispersity, to use your term. We don't know yet. It's too early.

DR. HAVEL: Dr. Rudel, in your summary slide, you talked about chemical potential changes, but you didn't comment on it in relation to the slide that showed the detailed composition as a function of particle size. What that did show was that the chemical potential of free cholesterol was much higher in the large particles than in the small particles and that could be looked at from the standpoint of the free cholesterol/phospholipid ratio which was at the bottom part of that slide and they were quite striking. So, it must have occurred to you, that the kind of thing that Chris Fielding talked about could be happening as an atherogenic factor, that there is a higher chemical potential in large particles. In relation to your two diets, is the chemical potential of unesterified cholesterol higher in the saturated fat for a given particle size than the polyunsaturated fat-fed animals?

DR. RUDEL: I don't remember the answer. We have that information though. I'll look that up for you. It doesn't strike me that it is particularly different, but I don't know for sure.

SPEAKER: Dr. Rudel, the transition temperatures for the cholesteryl ester that you showed, were those for the isolated cholesteryl esters or for the whole lipoprotein?

DR. RUDEL: Those were for the LDLs, the whole lipoproteins. John Parks did those on his differential scanning calorimeter and he actually used the column isolated LDL to do the DSC.

SPEAKER: I know from my own work and others too, the cynomolgus have very low HTGL levels compared to LPL which is quite distinct from humans. Do you happen to know the difference?

DR. RUDEL: Yes, I do. Actually, we've just recently looked at some of the hepatic triglyceride lipase and lipoprotein lipase levels in post-heparin plasma of these animals. Unexpectedly, the African green monkeys have a little bit higher hepatic triglyceride lipase level than the cynomolgus monkeys. They both have very high lipoprotein lipase levels compared to humans and the ratio is more like two to one lipoprotein lipase to hepatic triglyceride lipase in post-heparin plasma of these animals. It is quite different from humans. It is a very remarkable difference. The other point I'll make relating those two enzymes is that in heparin flushes of some of these livers we tend to find a lot of lipoprotein lipase in liver. I don't know the significance of that. You can't call it lipoprotein lipase with an antibody. I can just tell you its salt-inhibitable activity.

DR. FRANTZ: There's something that's bothering me a little bit about this postulated high atherogenicity of the larger particles. I have a feeling that Dr. Sniderman is going to tell us this afternoon about some small particles that contain a lot of protein and not much cholesterol that are especially atherogenic. Is there a contradiction there?

DR. RUDEL: There may be a contradiction. There may be two or more kinds. I would suggest that there might be several different properties of LDLs that are atherogenic and there might be commonality, depending on the form of lipoprotein particles secreted from the livers of the patients with hyperapobeta lipoproteinemia. The key difference that I've talked about in a review article recently is this triglyceride for cholesteryl ester exchange, the hypertriglyceridemia and how that affects--how intravascular metabolism has got to be so central in what we are looking at in terms of an end product particle here because everything that comes out of the liver doesn't have this composition. So, I'm not sure that the difference between the two situations isn't in part related to triglyceride metabolism. That is one possibility. Another possibility is that there are several kinds of LDL that are atherogenic. Ron Krauss's data suggests that there are several possible candidates.

SESSION VII. Genetics of ApoB-Containing Lipoproteins

Chair: Alan Chait

LOW DENSITY LIPOPROTEIN HETEROGENEITY IN FAMILIAL COMBINED HYPERLIPIDEMIA AND OTHER HYPERTRIGLYCERIDEMIC STATES

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Abstract

Low density lipoprotein (LDL) heterogeneity among normal and hyperlipidemic subjects has been recognized for many years, although the mechanisms involved in the formation and metabolism of different LDL are unknown. Factors that contribute to LDL heterogeneity include ambient triglyceride concentrations, the metabolic mileu in which the LDL exists, and genetic variations in the LDL itself. One can demonstrate interactions of these factors to determine LDL variability in a single individual.

Plasma low density lipoprotein (LDL) heterogeneity has been described among various lipoprotein phenotypes as well as within single individuals (1-8). Although it is unclear why different LDL subpopulations exist, several factors have been shown to influence the physical and chemical properties of LDL.

Plasma triglyceride (TG) concentrations have been shown to be correlated to the average size and density of LDL in normal and hyperlipidemic subjects (3,9-11). The association between size or density of LDL and plasma TG concentration is thought to be due to the exchange of very low density lipoprotein (VLDL)-TG for LDL cholesteryl ester (CE), mediated by lipid transfer protein (12). LDL-TG is then hydrolyzed, resulting in smaller, more dense LDL (12). This mechanism is consistent with the presence of small dense LDL described in hypertriglyceridemic (HTG) subjects in whom large pools of TG-rich VLDL would promote the exchange of VLDL-TG for LDL-CE (13,14).

Genetic factors may also influence LDL heterogeneity. Fisher et al. (15) have reported on the heritability of LDL molecular weight (MW). They found a high correlation between the mean LDL MW of 11 offspring and the mid-parental mean for their parents' LDL MW. Genotypic differences have also been seen in LDL isolated from subjects with familial combined hyperlipidemia (FCHL) compared to LDL isolated from normal controls (16). FCHL patients have smaller, denser LDL compared to the normal control subjects, independent of plasma TG concentrations (Fig. 1). Although genetic factors may play a role in influencing the physical and chemical properties of LDL, it is unclear what these factors are.

The metabolic behavior of LDL may also contribute to the variability in LDL MW, density, and composition as seen among normal and hyperlipidemia subjects. In familial hypercholesterolemia (FH), the fractional catabolic

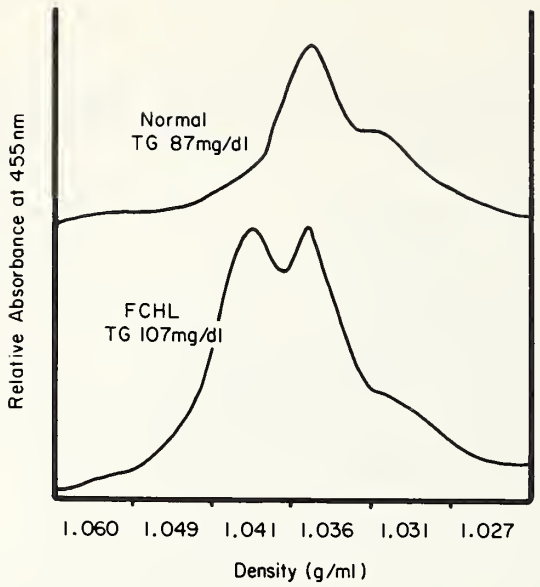

Figure 1. Densitometric scans at 455 nm of equilibrium density gradient profiles of LDL ($1.019<d<1.063$) by the methods of Krauss and Burke (7). The top profile is of LDL isolated from a normal subject and the lower profile is of LDL isolated from a subject with FCHL. Plasma TG concentrations are given for each.

rate (FCR) of LDL is decreased significantly due to a reduction in LDL receptors (17). This decrease in clearance is thought to contribute to the formation of large, buoyant, CE-rich LDL described in FH patients (18-21). Some of the differences in the size and density of LDL isolated from FH and normal subjects may be due to the influence of plasma TG concentrations (11). However, Patsch and co-workers (21) have shown that when LDL from FH and normal subjects within a single kindred were matched for MW, the LDL from the FH subjects contained more CE and less TG than LDL from the normal subjects. Therefore, it appears that at least 2 factors may be affecting the kind of LDL produced in FH: plasma TG concentrations and the metabolic behavior of LDL. Support for the latter observation comes from studies by Jadhav and Thompson (18) on the effects of plasma exchange on LDL composition in 7 patients with FH. Immediately after plasma exchange, the composition of LDL transiently resembled normal LDL, suggesting that the compositional abnormalities are due to the prolonged residence time of the LDL in the plasma. In contrast, small, dense LDL are seen in HTG subjects in which LDL plasma residence times are shorter than LDL from normal subjects (22,23). These data are consistent with the hypothesis that the catabolism of LDL, that is, the interaction of LDL with intravascular enzymes as well as LDL receptors, may influence its physical characteristics and contribute to LDL heterogeneity.

Kinetic heterogeneity of LDL has been suggested since the mid-70s (5,24, 25). More recently, the metabolic behavior of autologous LDL ($1.019<d<1.063$) was compared in subjects with FH and FCHL (26,27). In these studies, kinetic heterogeneity was required to describe the metabolic behavior of LDL in subjects with FCHL but not in subjects with FH. In FCHL, an additional plasma LDL compartment was needed through which the excess of LDL apo-B transport in FCHL was proposed to occur (26,27,28). Two recent studies have examined the metabolic behavior of various LDL subfractions isolated from HTG or hyperapo-

betalipoproteinemic patients (28,29,30). In both studies, the turnover rates of various LDL subfractions were different.

A single patient with FCHL was studied in the metabolic ward in Seattle on two diets (saturated vs. polyunsaturated fat) which led to different lipoprotein phenotypes. On the saturated fat diet, the patient was HTG (348± 33 mg/dl; mean±SD) and had a relatively homogeneous dense population of LDL as described by flotation gradient ultracentrifugation (Fig. 2). When the LDL

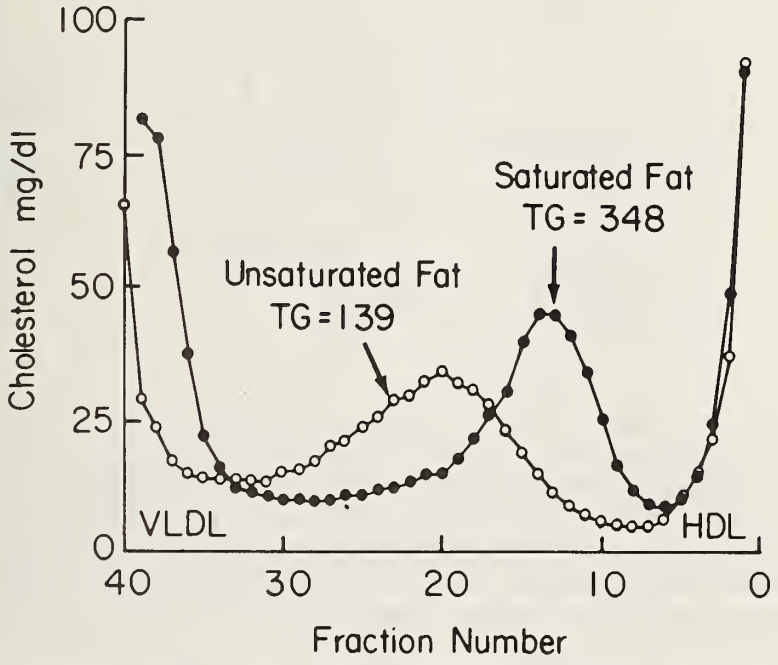

Figure 2. Distribution of lipoproteins in whole plasma using nonequilibrium density gradient ultracentrifugation in a vertical rotor as described by Chung et al. (31). Cholesterol was measured in 40 fractions collected across the density gradient, which were designed to highlight low density lipoproteins. The LDL peak in the saturated fat diet was relatively narrow, while that in the unsaturated fat diet was broader and more buoyant.

was isolated, radiolabeled, and reinjected into the subject while consuming the saturated fat diet, 95% of the LDL was distributed in the plasma compartment which was unique for FCHL (see model B in ref. 26) (Fig. 3A). Plasma TG concentrations were then lowered (139±15 mg/dl) by placing the subject on a diet rich in polyunsaturated fat. During this time, LDL cholesterol concentrations increased and the LDL appeared more buoyant and heterogeneous based on its flotation characteristics (Fig. 2). The shift in the apparent density of the LDL is consistent with the decrease in TG concentrations (11). When the metabolic behavior of LDL was examined in this subject while consuming the polyunsaturated fat diet, the kinetic model suggested that LDL mass was distributed into 2 plasma pools (Fig. 3B),consistent with the more heterogeneous appearance of the LDL by density (Fig. 2). The coordinated changes seen in the lipoprotein phenotype, the LDL flotation characteristics, and the kinetic models support the hypothesis that both genotype and TG concentrations contribute to LDL heterogeneity.

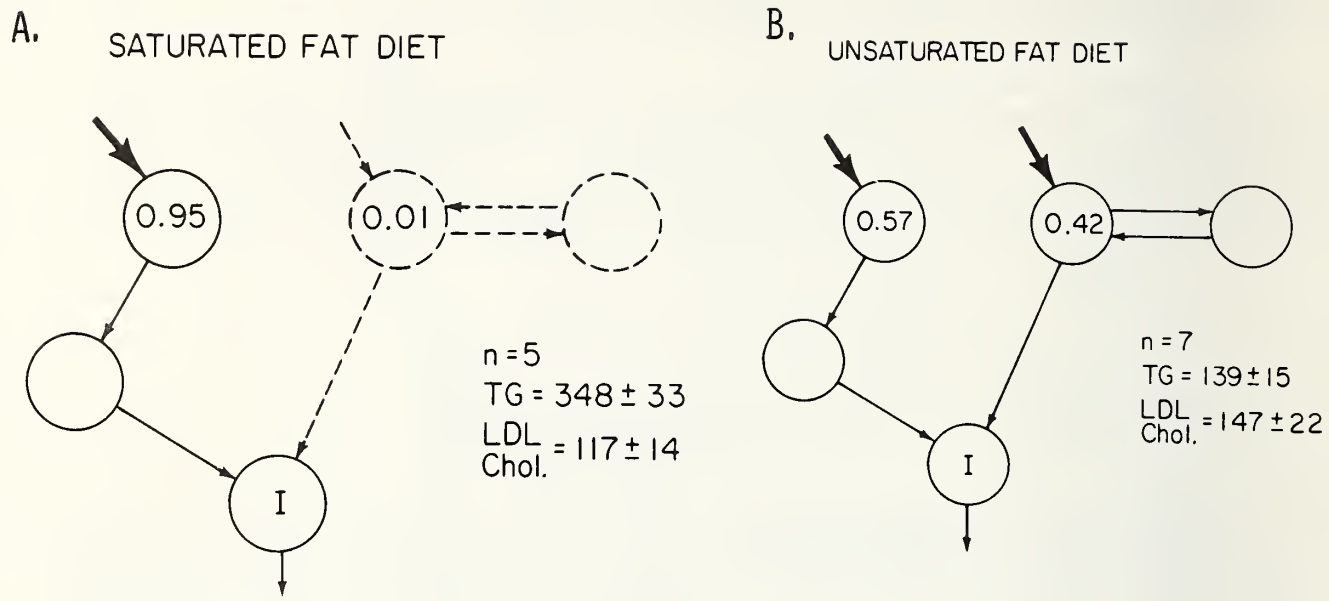

Figure 3. Model B for LDL apo B metabolism as described by Foster et al. (26). This model is characterized by two plasma compartments (those which contain numbers) representing kinetically different subfractions of LDL. On the saturated fat diet, 95% of the LDL apo B mass is in a rapidly turning over plasma pool consistently seen in FCHL (28). With lower TG levels, the LDL mass was distributed more evenly between this compartment (57%) and the compartment usually seen in normal individuals (42%).

As reviewed above, plasma TG concentration, metabolic behavior, and genetic factors may contribute to the differences in LDL size and density among various lipoprotein phenotypes. However, very little is known about the mechanisms involved in the formation of various LDL subfractions within an individual LDL sample. LDL polydispersity has been described in a number of hyperlipidemic (Type IIa, IIb, IV, and V) and normolipemic human subjects using analytical, density gradient (Fig. 4), and/or zonal ultracentrifugation (1-4,6-8). Recently, Krauss and Burke (7) have characterized LDL density and size heterogeneity in normolipemic subjects using analytical and equilibrium density gradient ultracentrifugation and gradient gel electrophoresis. These studies have emphasized the complexities of LDL heterogeneity by demonstrating both size and density variability within an individual LDL sample.

The compositional characteristics of different LDL subfractions have not been examined extensively. In studies that have examined the chemical composition of various LDL density subfractions (1,16,32), with increasing density there is a decrease in cholesteryl ester to protein ratio, reflecting a decrease in core lipid. Very little is known about the apoprotein distribution among different LDL subpopulations. Lee and Alaupovic (32) have demonstrated slightly variable immunoreactivity to apo C among different LDL density subfractions, but they did not measure any other non-apo B apoprotein. More recently, Gibson and coworkers have described the presence of variable amounts of apo E and apo CIII in LDL isolated by gel filtration (33). The only detailed studies on the distribution of apoproteins among different LDL density subfractions were reported in human umbilical cord blood LDL (34) and

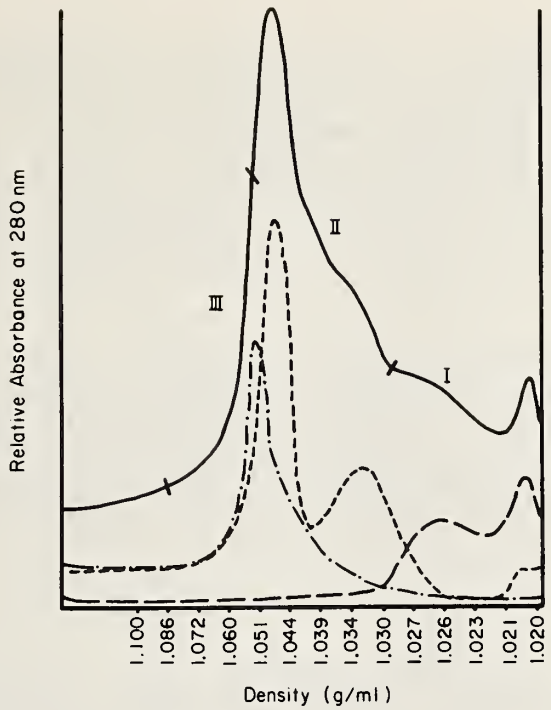

Figure 4. Density gradient profiles of 1.006<d<1.063 lipoproteins isolated by sequential ultracentrifugation from a subject with FCHL. The lipoproteins were underlayered into a discontinuous salt gradient consisting of 5.2 ml of a 1.006 g/ml KBr solution, 4.0 ml of a 1.063 g/ml KBr solution, and 2.5 ml (including the lipoprotein sample) of a 1.21 g/ml KBr solution and subjected to ultracentrifugation in an SW-41 rotor for 24 hr at 41,000 rpm at 15°C. After centrifugation, each sample was drained from the top of the tube by pumping a dense solution (Fluorinert; 1.85 g/ml) into the bottom of the tube. The eluent was drained through a UV monitor and into a fraction collector. The profiles were obtained from recording absorbance at 280 nm. The refractive index was measured at 15°C on alternate fractions collected from a tube containing the same salt gradient but without a sample. The profile of the starting material is shown in the top profile ( ). The material within the regions between the solid lines defining fraction I, II, and III was pooled and subjected to DGUC by the same procedure used for the initial starting material. Fraction I ( ) - 1.023<d<1.034; fraction II ( ) - 1.034<d< 1.0445; fraction III ( ) - 1.0445<d<1.083. At least two kinds of density subpopulations can be seen in each fraction.

in two species of nonhuman primates (35). In both studies, the ratio of apo E:apo B was highest in the more buoyant subfractions and decreased with increasing density, while the ratio of apo AI:apo B increased with increasing density. We have also seen similar apoprotein heterogeneity among LDL density subfractions in subjects with FCHL (Marzetta, CA and Brunzell, JD, unpublished observations). It is still unclear, however, how much of the non-apo B apoproteins are due to the presence of remnant-like or HDL-like particles or whether distinct apoprotein heterogeneity exists within LDL subpopulations.

Variations in the structure of apo B have been identified using monoclonal antibodies (36-38). The epitopes expressed on apo B have been shown to be correlated to the size, density, and lipid composition of LDL (36,37).

It has been hypothesized that the influence of size or density of various LDL subpopulations on the conformation of apo B may affect its recognition by the LDL receptor and thereby alter its metabolic behavior (37). Additionally, genetic polymorphism has been described in apo B in the general population (38,39). It is speculated that structural differences in apo B will be found that will directly influence the kind of LDL produced and/or indirectly influence the formation of LDL subpopulations by affecting their metabolic behavior.

In summary, plasma TG concentrations, genotype, and the metabolic behavior of LDL may contribute to the differences in LDL seen among normal and hyperlipidemic subjects as well as within an individual. Although the atherogenic properties of different LDL subfractions are poorly understood, it is important to continue to examine mechanisms that influence the formation and regulation of LDL for potential intervention and treatment of coronary artery atherosclerosis.

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HYPERAPOBETALIPOPROTEINEMIA AND LDL AND HDL_2 HETEROGENEITY

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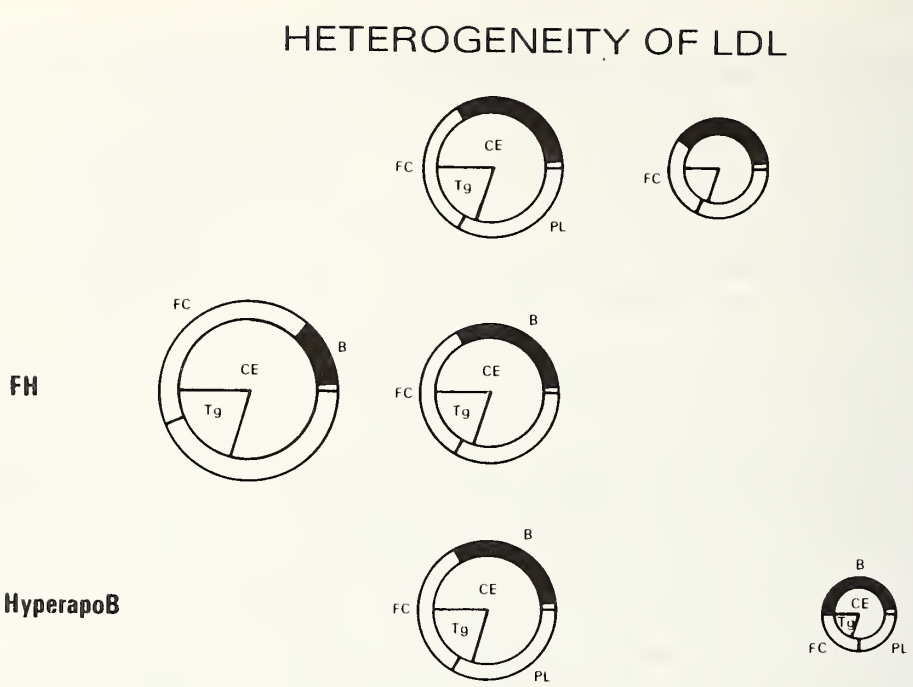
Introduction

Hyperapobetalipoproteinemia (HyperapoB) and Familial Hypercholesterolemia (FH) share a number of features in common. Both are associated with premature coronary artery disease and in both, more apoB than normal is synthesized (1). As a consequence, in both, the number of LDL particles per ml of plasma is markedly increased and as a consequence of the increased LDL particle number, the entry rate of LDL into arterial wall is likely markedly increased. As a consequence of that, premature arteriosclerosis results. Though this sequence may hold for both, the low density lipoproteins (LDL) in FH differs markedly from the LDL in HyperapoB (2). In FH, LDL cholesterol levels are markedly elevated and the normal clearance of LDL severely impaired. In HyperapoB, LDL cholesterol is normal or near-normal while LDL apoB is clearly increased and clearance of LDL is normal (1,3). In FH, the ratio of cholesterol to apoB in LDL in much higher than in normals, while in HyperapoB, this same ratio is much lower than in normals (2). These differences between disease and normal represent one way in which the variability of LDL composition may be expressed. Our purpose here is to examine the physicochemical basis for LDL heterogeneity with particular regard to normals and HyperapoB. Once the physical model to accomodate these differences is presented, we will then try to explore the dynamics responsible both for the variation in LDL composition among LDL particles within normals as well as the variations responsible for the characteristic differences in mean LDL composition between HyperapoB and normals. We believe the physical basis for the difference in LDL composition is now evident and the kinetic basis likely. In brief, LDL particles can be extensively remodelled where this remodelling is a two step process consisting in the first instance of a cholesterol ester (CE) - triglyceride (Tg) exchange such that CE leaves the LDL core and Tg enters it. The second step is hydrolysis of the Tg with the two steps in tandem producing the net result of an LDL particle depleted in core lipid and relatively enriched in protein.

LDL Heterogeneity In HyperapoB and In Normals

HyperapoB is defined as an increase in the plasma level of LDL apoB in the face of a normal or near-normal LDL cholesterol (3). We have shown by a variety of physical techniques including equilibrium density ultracentrifugation, analytic ultracentrifugation and electron microscopy that in HyperapoB most LDL particles are smaller and denser than normal. The principle change in LDL composition occurs in core lipid content with the denser LDL particles having less CE, the more buoyant LDL particles having more (2). There is thus a direct relation between LDL cholesterol ester content and LDL buoyancy and also a direct relation between LDL core lipid content and LDL molecular weight.

Figure 1



In any normal individual, LDL particles are not uniform in their makeup. Some are larger and contain more cholesterol; others are smaller and denser and contain less. These changes are all consistent though with a spherical model of an LDL particle. This model states that LDL particles are spheres made up of a core surrounded by a coat. The most non-polar lipids, CE and Tg, make up the core, while the coat which surrounds it consists of free cholesterol, phospholipids, and virtually a single protein - apoB.

LDL particles do vary in size but it is the core which changes in diameter, not the coat since the thickness of the coat is always the same, 22.5 A°, the width of a phospholipid bilayer. Further all LDL particles seem to have the same amount of apoB winding through the coat. Thus as LDL particles change in size, the volume of core lipid per particle changes dramatically. For example, an LDL particle 150 A° in diameter will contain only 15% of the volume of core lipid found in an LDL particle 250 A° in diameter. Since most of the core lipid is CE, there are obviously remarkable differences in the LDL cholesterol to apoB ratio in LDL particles of different size. This is not to say there are no changes in the makeup of the coat as LDL particles vary in size. There are, both in terms of lipid to protein ratio and the configuration of apoB (4). But these changes are relatively small and are a consequence not a cause of the major changes in the volume of core lipid.

How Then Do LDL Particles Differ Amongst Themselves?

We believe the scheme shown in Figure 1 explains most observations. First, even in normals, there is a spectrum of LDL particles, some larger because they contain more core lipid - that is more CE; others smaller because they contain less. Because the amount of apoB per particle is constant, the smaller particles are denser, the larger particles more buoyant. Thus in any normal, within the LDL

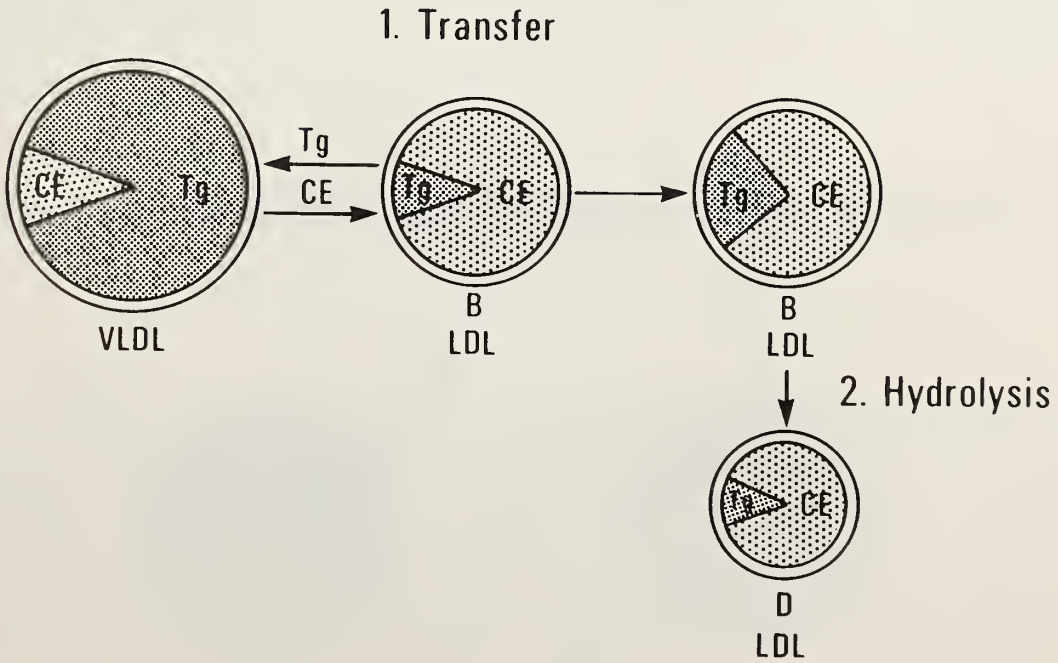
density range, there is variation in the makeup of the LDL particles but the variation is predictable. LDL particles vary from buoyant to dense, from larger to smaller, from particles with considerable core lipid to particles with much less (2,5,6). Nevertheless even though there is a continuum in terms of LDL composition, LDL particles are not distributed uniformally throughout that continuum. In normals, most particles, in fact, are found within quite a narrow portion of the LDL density range. Such LDL is in Fisher's phrase, monodisperse (7).

But these differences in LDL within an individual are only one way in which LDL is heterogeneous. As we have already noted, disease stamps even more dramatic alterations on LDL makeup. Though more dramatic however, these changes can still be related to the same scheme of LDL structure. Thus in FH, the mean LDL cholesterol to apoB ratio is increased because there is an important subfraction of LDL whose content of CE is markedly increased (2). By contrast, in HyperapoB most of the LDL particles are denser and smaller than normal because they are depleted in core CE but contain a normal amount of apoB (2). From this come two maxims: first, since the apoB content per particle is constant, LDL apoB levels are a reasonable index of the number of LDL particles per ml of plasma; second, since the cholesterol content of an LDL particle can vary so widely, LDL cholesterol levels are only a very rough guide - indeed often a frankly inaccurate one - to LDL particle number in plasma.

What Then Accounts For These Differences In LDL Composition?

The answer appears to be a two step process with first Tg replacing CE within the core of an LDL particle and then the Tg itself being hydrolyzed. That core lipid exchange can occur is now widely accepted. That such exchanges are important features of LDL metabolism is much less widely appreciated. Zilversmit and his colleagues were the first to demonstrate not only that the non-polar core lipids could exchange between two lipoproteins, but for this to occur, a specific transfer protein must be present (8). Early on, it was argued that the protein was one of the apoproteins, apoD (9), but this now seems unlikely and the protein is generally referred to as cholesterol ester transfer protein (CETP).

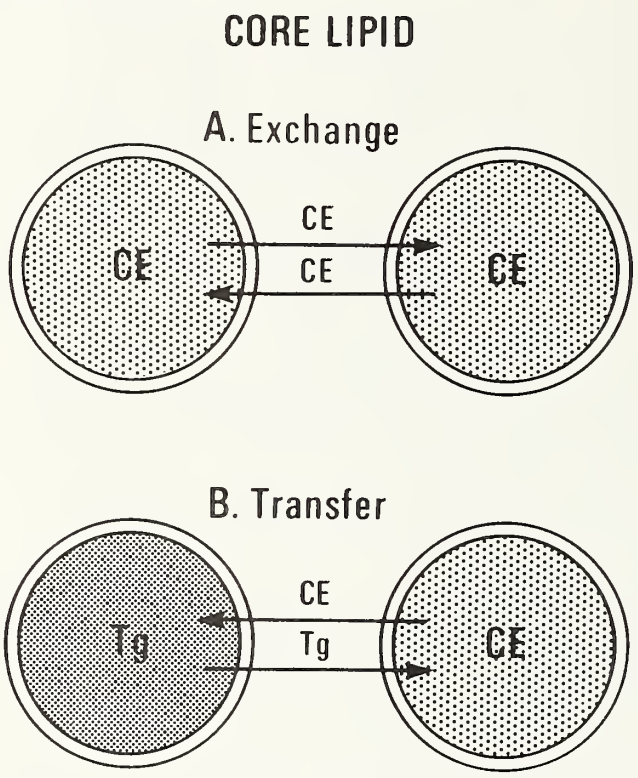
Figure 2



LDL remodelling therefore probably occurs much as shown in Figure 2. First, CETP binds to LDL; then there is transfer of CE from buoyant LDL to VLDL, with in turn, transfer of Tg from VLDL to LDL. By these transfers, the LDL core is initially unchanged in size but enriched in Tg and depleted in CE. But the core Tg is then vulnerable to hydrolysis by either lipoprotein lipase or hepatic lipase. When this second phase occurs, the volume of core lipid is reduced, producing a smaller, denser LDL particle.

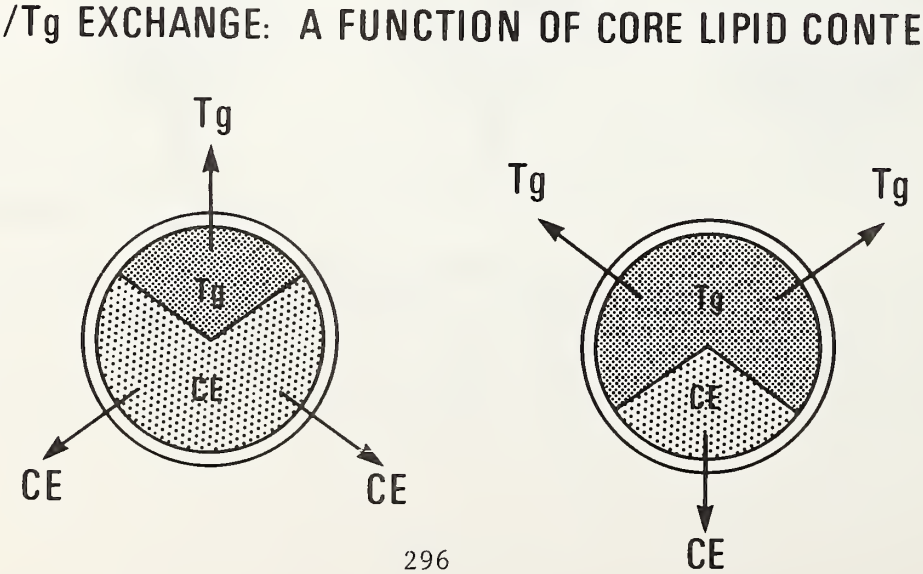
The rate of such remodelling obviously depends on a number of factors which include: 1) the number of acceptor and donor particles; 2) the mass of CETP; 3) the phospholipid and fatty acid content of the lipoproteins since these will influence CETP binding (10); and 4) the relative composition of core lipid. This last factor may be worth amplification.

Figure 3



If as shown in Figure 3, two CE rich lipoproteins collide with CETP, there will simply be exchange of CE molecules without any net change in composition. It is only the interaction of a Tg rich particle with a CE rich one that allows remodelling to emerge.

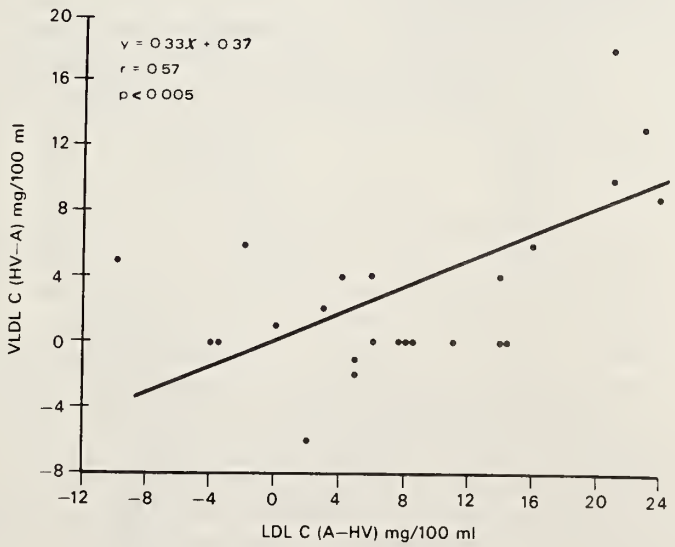
Figure 4



Thus as shown in Figure 4, the relative content of core CE in Tg within any particular donor particle will determine the extent of this process. If one particle has a mixed core but mainly CE, and this particle interacts with a CE lipoprotein, then there will be predominantly reassortment of CE with little net transfer. If the core is predominantly Tg however, there will be mainly net transfer with extensive remodelling within the acceptor particle. These predictions are indeed confirmed by experimental data with CE-Tg exchanges greater when HDL is incubated with chylomicrons than when it is incubated with VLDL (34) and with greater exchange as well when HDL is incubated with nascent rather than plasma VLDL - the former having less core CE than the latter (35).

These sequences can be demonstrated in vitro therefore. But it should also be noted, however, that the essential elements have also been demonstrated in vivo. For example, we have observed in man (13) that LDL isolated from the hepatic vein contains less CE but more Tg than arterial LDL indicating that within the splanchnic bed, CE is removed from LDL and Tg added to it. As well, and not surprisingly since VLDL is synthesized in the liver, hepatic vein VLDL cholesterol was higher than arterial VLDL cholesterol. What was not anticipated however was the linear relation observed between cholesterol removed from LDL and cholesterol appearing in VLDL (Figure 5). That is, the more cholesterol removed from LDL during its passage across the splanchnic bed, the more the VLDL cholesterol rose. Figure 5 displays data from 24 patients showing for the group a significant relation between the two.

Figure 5: Changes in lipoprotein composition across the splanchnic bed.



To understand why this may occur, one must appreciate that between 20 and 40% of all LDL particles are present in an extravascular pool, and the major site of this extravascular pool is around the hepatocyte. This is certainly the case in the pig, and very likely the case as well in man (14). Given that, the following sequence seems most probable: since ACAT activity is low in the human liver (15), nascent VLDL has little or no CE in its core. Further, data to be shown later make it likely that buoyant LDL is the dominant LDL subspecies present in the hepatic extravascular space. Thus nascent VLDL collides with buoyant LDL to which CETP has

bound and the two-step LDL remodelling process begins. Since hepatic vein LDL Tg is increased, it is likely that much of the subsequent hydrolysis occurs due to the action of peripheral lipoprotein lipase. However it is also possible for this to occur right in the liver itself due to hepatic lipase. And indeed, the complete remodelling of LDL might well explain the apparent direct production of dense LDL in normals and HyperapoB (1). Further, if IDL can also participate in the CE-Tg exchanges in the liver, rapid remodelling while in the liver bed might then explain why total IDL apoB production appears to be less in turnover studies than total LDL apoB production. That is, if an IDL or buoyant LDL particle is very quickly remodelled, it may never enter the plasma compartment which is the only compartment sampled in such studies.

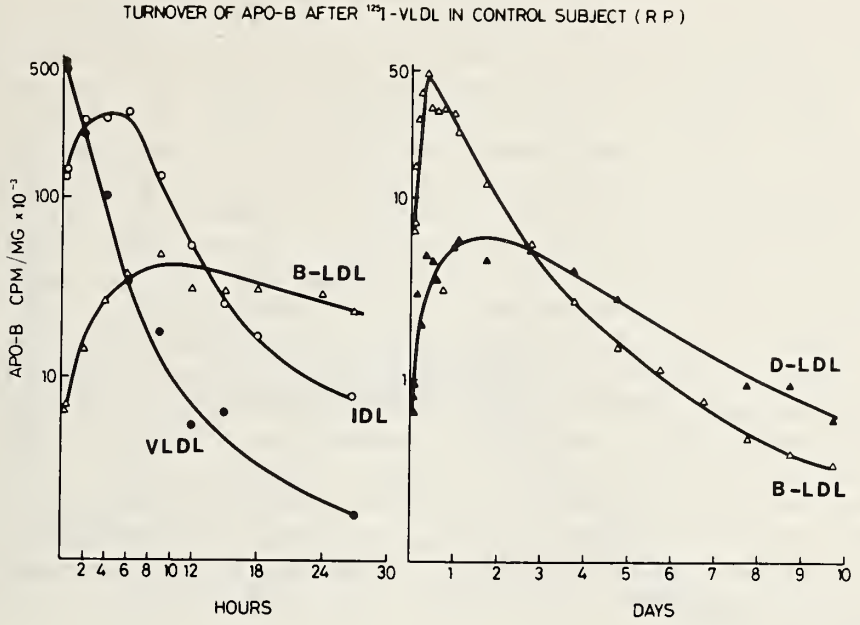
Obviously, CE-Tg exchanges are not necessarily restricted to the perihepatocyte space, and they could occur elsewhere in the vascular space. Emphasis has been placed on the liver because our in vivo data support this concept and as noted above these exchanges should be largest here. Under certain circumstances however, exchange may reach important proportions elsewhere. These include, as will be discussed, the postprandial period and as well certain cases of hypertriglyceridemia. With regard to the latter, if chylomicron or VLDL levels were high and LDL particle number low, then there might well be considerable remodelling of LDL due just to random collisions in the peripheral circulation as well as in the liver bed. This phenomenon probably explains why the LDL cholesterol to B ratio is not a simple function of the plasma Tg level and thus why it may be low when the LDL apoB is also low (16).

The Impact of HyperapoB on CE-Tg Exchanges

In HyperapoB, the same phenomena occur both in the liver and the peripheral circulation but are amplified. As such they impact even more forcefully on LDL composition, and indeed are so pronounced, that HDL_2 composition may be altered as well. Thus in HyperapoB, hepatic apoB production is increased such that in any unit time, the number of VLDL particles leaving the hepatocyte and thus able to be Tg donors is increased by about 200%. At the same time, LDL particle number is increased - but only by 50 to 100%. Thus at the crucial site of interaction, both lipoproteins are present in increased numbers with nascent VLDL even more abundant than buoyant LDL. As a consequence, CE-Tg exchanges are exaggerated such that more of the LDL core CE is replaced by Tg, and as a consequence denser smaller LDL depleted in CE and relatively enriched in protein is produced.

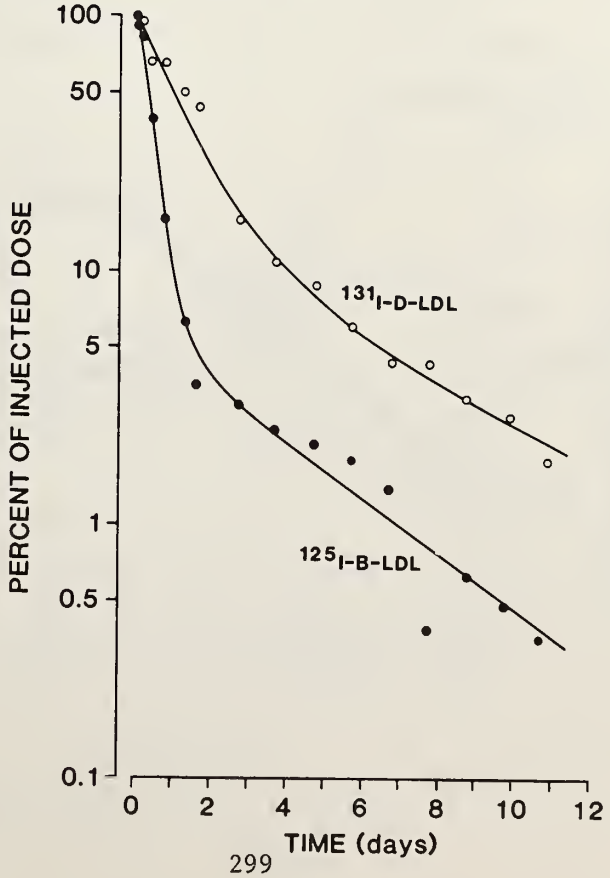
However LDL particles differ not just in their composition but in their metabolic fate as well. There is thus a temporal as well as structural order to LDL heterogeneity. As shown in Figure 6, apoB turnover studies we have performed both in normals and in HyperapoB (1) demonstrate unequivocal precursor-product relationships between VLDL and IDL, between IDL and buoyant LDL, and between buoyant LDL and dense LDL. Thus dense LDL is remodelled buoyant LDL, not a de novo product. This is the case both in normal and HyperapoB but not in FH. As already noted, rapid remodelling may occur in the splanchnic bed and this may well explain the apparent direct production of buoyant LDL which does not pass through IDL and the appearance of dense LDL which does not appear to pass through buoyant LDL. Thus the cascade from buoyant to dense LDL is clear. More recent studies that we have performed using multicompartmental modelling indicate that that the reverse sequence may occur as well - namely a portion of the dense LDL can reacquire lipid and become buoyant again. This process remains to be studied in detail but appears to be considerably smaller in extent than the initial cascade pathway described.

Figure 6



Not only is buoyant LDL a precursor of dense LDL, but its relation to the liver and its metabolic fate differs from dense LDL. This can be inferred from their respective plasma decay curves (Figure 7). Note that for buoyant LDL, the curve is clearly biexponential whereas for dense LDL it is close to monoexponential (Figure 7). Given that the initial exponential represents reversible equilibration with the extravascular space, our data indicate that it is predominantly buoyant not dense LDL that is found about the hepatocyte and the clearness of the distinction suggests that the buoyant LDL is not simply free within the space of Disse but is rather in some close association with the hepatocyte itself.

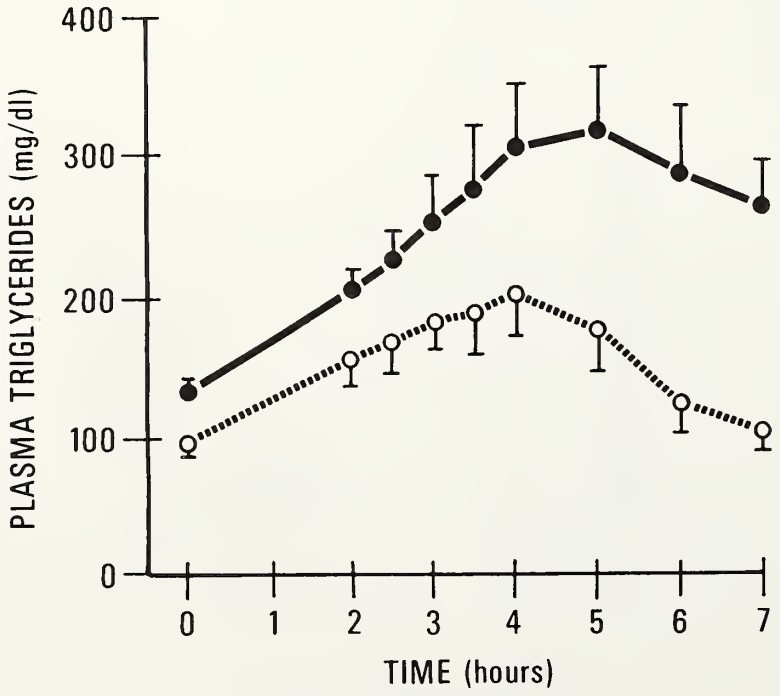
Figure 7



We have shown that the fractional clearance rate of dense LDL is much less than buoyant LDL both in normals and HyperapoB - an observation which on the face of it suggests that dense LDL might bind less well to the LDL receptor than light LDL. In support of this hypothesis, apoB conformation does appear to alter as LDL particles become smaller. And indeed, if apoB is conserved within the particle, this could hardly be otherwise for the same amount of protein now must extend over a much smaller circumference. That at least is the explanation we believe for our observation of altered immunoaffinity of some but not all apoB epitopes as LDL particle size changes (4). Further, of the three epitopes, whose immunoaffinity diminished as LDL particle size decreases, two are located near to the probable receptor binding region of apoB. As a consequence, it is possible that dense LDL might bind less avidly than buoyant LDL to the BE receptor, and so explain the lowered fractional catabolic rate. Indeed, Eisenberg et al (17) have shown that dense LDL isolated from hypertriglyceridemic patients was bound and degraded by cultured skin fibroblasts substantially less well than normal LDL - observations which would appear to support this concept. In our hands, however, these differences between LDL particles have been less clear cut. To be sure there has been a tendency for dense LDL to bind less well than buoyant LDL, but over a 24 hour incubation period, no difference in degradation was observed. In addition, the day to day variability in the binding assay itself makes, we believe, the likelihood of reaching definitive conclusions remote.

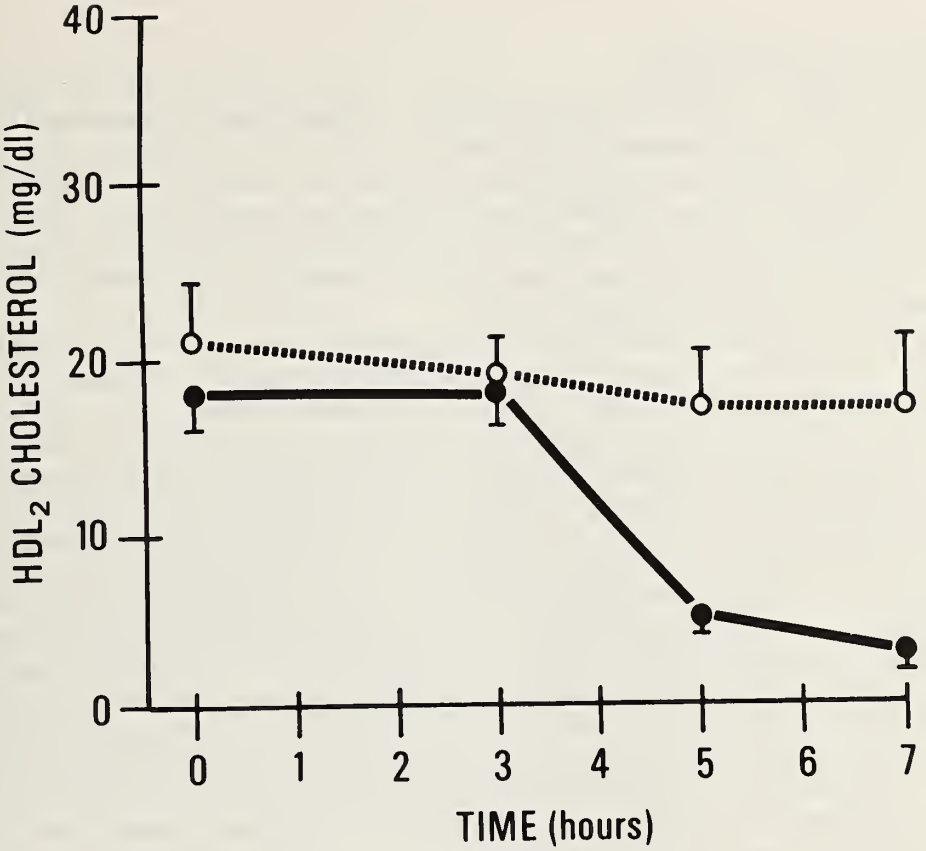
Certainly the interpretation of a fractional catabolic rate calculated from a plasma decay curve is not as straightforward as it might appear. For example, there are three fates open to buoyant LDL: 1) it could be degraded irreversibly either by the specific or non-specific pathways; 2) it can leave the vascular space, enter the extravascular exchangeable pool, and then reenter plasma; and 3) presumably after 2, it can then enter the hydrolytic cascade and become dense LDL. Dense LDL, on the other hand can leave plasma irreversibly, or as our more recent turnover studies have shown, at least a portion may reacquire lipid and become larger and more buoyant again. Thus the fractional catabolic rate of buoyant LDL includes both irreversible degradation of the particle and conversion to dense LDL.

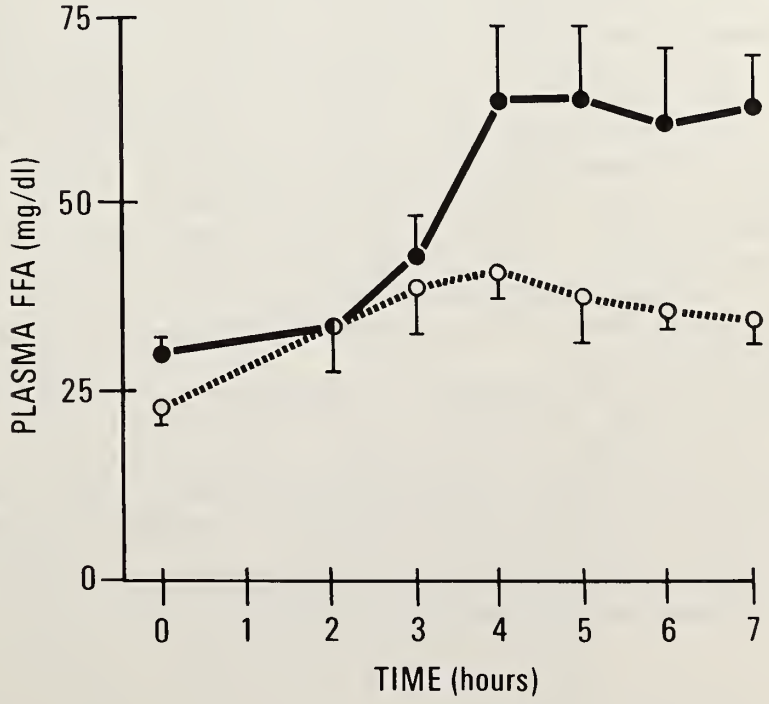
Figure 8: Lipoprotein changes following an oral fat load.



Normals in O circles. HyperapoB solid circles.

Figure 9 & 10: Lipoprotein changes following an oral fat load.





Normals in O circles. HyperapoB in solid circles.

HDL_2 Heterogeneity In HyperapoB

Peripheral CE-Tg exchanges are likely also exaggerated in HyperapoB. In addition to VLDL levels which are frequently elevated in HyperapoB, chylomicron levels remain higher than normal following an oral fat load even in patients with HyperapoB who have normal fasting Tg levels (18). That is, following an oral fat load, plasma Tg levels rise higher and remain elevated longer in patients with HyperapoB than normals, chylomicrons accounting for most of this difference. CE-Tg exchange should certainly occur between chylomicron and LDL during this period but also almost certainly involves HDL_2 as well.

That at least is what we believe underlies the sharp drop in HDL_2 cholesterol levels observed in patients with HyperapoB after an oral fat load (18). It should be noted that this drop does not occur immediately but only after a period of several hours. It also does not seem to be really a function of plasma Tg level and it did not occur to any marked degree in hypertriglyceridemic patients without HyperapoB. One key to the process may be simultaneous plasma free fatty acid (FFA) levels. Tall and coworkers (19) have shown that CETP binding to lipoproteins and as a consequence the rate of CE-Tg exchange is a function, in vitro at least, of ambient FFA levels. Postprandial FFA levels in HyperapoB patients rose higher than in normals and so would be expected to increase CE-Tg exchange with remodelling of both LDL and HDL_2. Thus a drop in HDL_2 cholesterol levels during the postprandial period most likely represents replacement of cholesterol by Tg within these particles. Parenthetically we should note that CED-Tg exchange might underlie, at least in part, the inverse relationship between HDL_2 and cholesterol concentration and the risk of CAD rather than impaired reverse cholesterol transport. Further the increased FFA levels in HyperapoB could be a consequence of the impaired intracellular metabolism of fatty acids we have observed with this syndrome (20). Thus if adipose tissue FFA uptake and incorporation into Tg were reduced in HyperapoB, then extracellular FFA levels would rise, possibly inhibit lipoprotein lipase, and so produce prolonged postprandial chylomicronemia.

Both buoyant and dense LDL should enter arterial walls but whether their metabolic fates there differ remains completely unknown. It is obviously important though to discover whether their impact on a variety of cells types differs to any important degree. The proclivity to premature atherosclerosis in HyperapoB seem likely, at least in part, to be due to the increased particle number in this disorder. However the possibility that dense LDL may be more atherogenic than buoyant LDL certainly cannot be dismissed at this point.

There thus seems to be a close connection between the static and dynamic aspects of LDL heterogeneity. LDL heterogeneity is a consequence of the normal metabolism of LDL. In HyperapoB, the increased VLDL apoB synthesis exagerates and amplifies these processes, and as a consequence, produces markedly but characteristically altered LDL particles.

One final comment may be worthwhile. Why have the differences in LDL composition which have been known so long been so far ignored? There is, obviously, no certain answer to this question but it seems likely that it is in large part the potential relationship to disease that has caused recent interest in this area to burgeon. We should point out that HyperapoB was recognized by a study of patients with premature coronary atherosclerosis; because increased apoB levels appear to be associated with premature atherosclerosis, understanding their pathogenesis and consequences would seem to be of the first importance.

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ALLOTYPE EXPRESSION AS MEASUREMENT OF APOLIPOPROTEIN B HETEROGENEITY

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Introduction

The introduction of monoclonal anti-apolipoprotein B (apoB) antibodies as probes of lipoprotein and apolipoprotein structure has provided means for analysis of subtle differences in lipoproteins. Basically, there appear to be two kinds of antibodies. Many antibodies are directed against epitopes common to all forms of human apoB. They react uniformly with apoB obtained from different individuals and different families. On the other hand, some antibodies detect individual differences in antigenic specificity of apoB. The occurrence of such differences in apoB within a species is called **allotypy** (1) and the different immunophenotypes are termed **allotypes**. The present paper summarizes studies carried out in Finland using both types of antibodies. In a recent study (2) employing an allotype-specific antibody (MB-19) it was shown that this antibody was directed against Ag(c), an alloantigenic determinant previously described using human antisera (3). This provided proof that true genetic polymorphisms, such as the Ag polymorphism, can be conveniently monitored using monoclonal antibodies.

Antibodies Used in the Detection of ApoB Allotypes

Human antisera. Some patients who have received multiple blood transfusions produce antibodies directed against antigens located on low-density lipoprotein (LDL)(4). These so-called Ag antigens are almost certainly located on apoB and constitute five pairs that behave like products of allelic genes (5). Such human Ag antisera have proved useful in the characterization of the Ag allotypic system and in establishing the co-dominant mode of transmission of Ag antigenic determinants. However, the limited supply of these reagents has allowed only a few centers to carry out studies. Thus, the relation of the Ag polymorphism of LDL to lipid levels and atherosclerosis remains, except for one study (6), unexplored. This shortcoming may be soon overcome as monoclonal anti-apoB antibodies take over the task of detecting Ag antigenic sites (See below).

Allotype-specific monoclonal antibodies. Monoclonal antibodies detect subtle differences in closely related proteins. Theoretically, it is to be expected that monoclonal antibodies would be created that recognize the Ag antigenic sites. In a

recent paper (2) we have demonstrated that an antibody produced by Curtiss and Edgington (7) was able to distinguish between two such antigenic sites, the Ag(c) and Ag(g) epitopes. An early report using the same antibody (8) inaccurately stated that the Ag system was unrelated to the polymorphism detected, but the authors have re-evaluated their method and confirmed that the polymorphism defined by the antibody and the Ag(c/g) polymorphism are identical (9).

Pan-apoB antibodies. Some antibodies appear to react uniformly with apoB from different individuals and may be called pan-apoB antibodies (10). Antibody 2a (11) provides a typical example of such a pan-apoB antibody. It recognizes a domain on apoB which is close to the LDL receptor recognition site (11), which occurs in every LDL molecule (12), and belongs to an ancestral part of the molecule which has been conserved during evolution (13). Having tested more than a thousand plasma or LDL-apoB samples from different individuals we have not observed significant differences in reactivity with this antibody. Nevertheless, slight but significat differences can be distinguished by this antibody between LDL-apoB and VLDL-apoB in the same individual, suggesting that even the universal epitope defined by antibody 2a is modified by the surrounding lipoprotein structures (14). Pan-apoB antibodies are useful reference antibodies in assays utilizing allotype-specific antibodies for polymorphism detection (2,15). Variation in reactivity with a pan-apoB antibody usually results from a damaged sample or inaccurate assay alerting the researcher to check the system.

Assay Method for ApoB Allotype Detection

Competitive displacement assays provide the most sensitive method for detecting allotypic variation of apoB (2,15). Studies carried out in Finland (2) are based on the following. A standard preparation of LDL is immobilized in microtiter wells (solid phase). Both antibody and a standardized amount of sample apoB are added to the wells thus making sample apoB to compete with the immobilized LDL-apoB for the antibody. Depending on whether sample apoB binds to the antibody with high, intermediate or low affinity, it will cause a great, intermediate or small degree of displacement of antibody from the immobilized standard apoB. Washing of the wells removes all antibody not bound to the immobilized apoB. The amount of this solid phase-bound antibody is quantitated by a second (anti-mouse) antibody using ELISA. Typically, allotype-specific antibodies produce curves reflecting the different binding affinities while pan-apoB antibodies produce superimposable curves (Fig. 1).

Distribution of ApoB Allotypes in Families

Assays carried out in Finland (2) and elsewhere (15) employing the allotype-specific antibody MB-19 invariably demonstrated that apoB expressed one of three binding patterns or

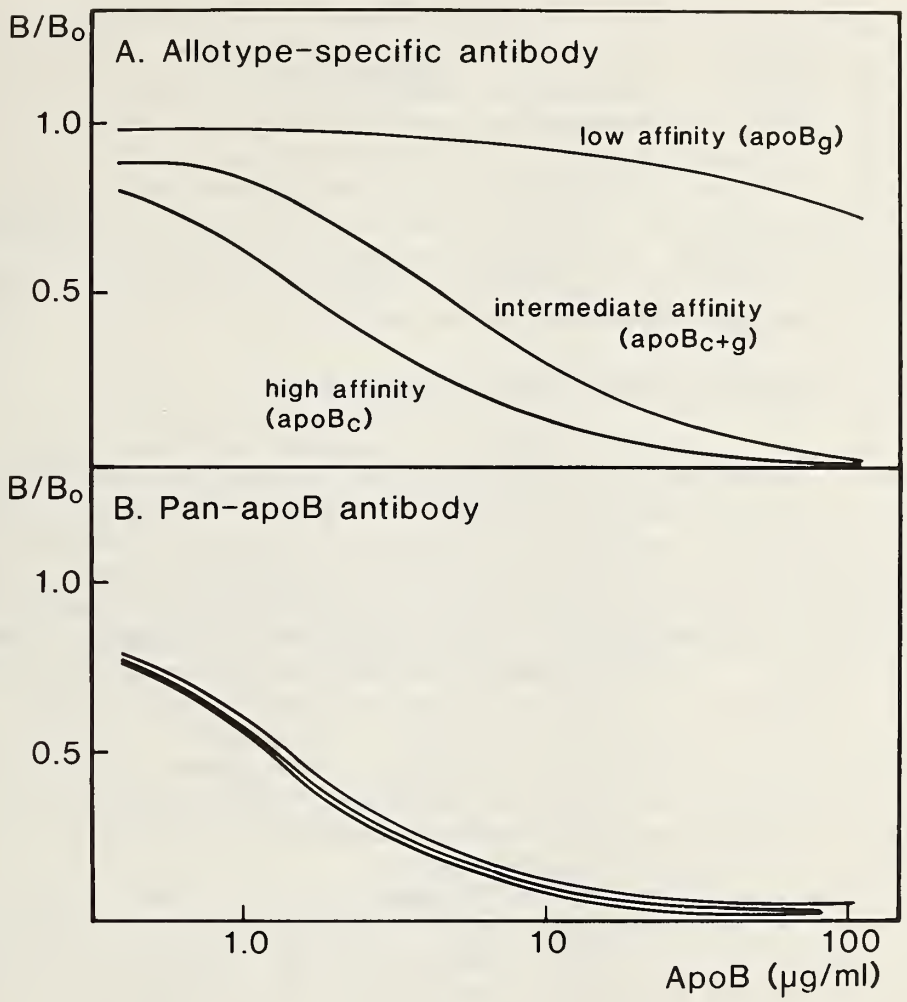

Figure 1. Samples of apoB obtained from different individuals produce different types of displacement curves when assayed with allotype-specific antibodies (A) but superimposable curves with pan-apoB antibodies (B).

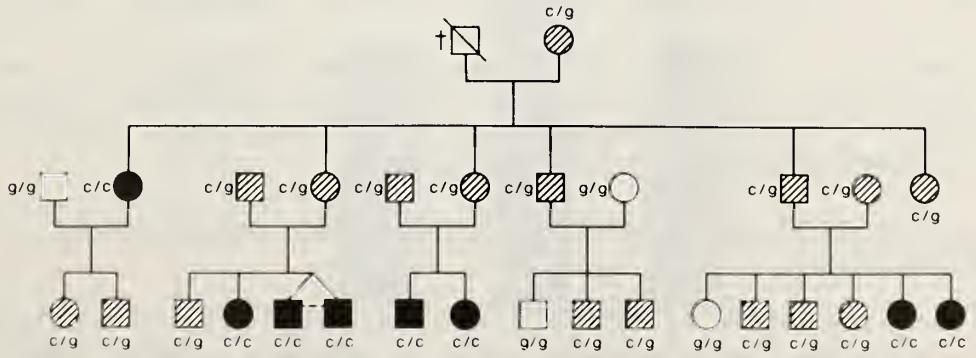

Figure 2. Distribution of ApoB binding pattern with antibody MB-19, and Ag genotypes in a Finnish kindred (2). Black symbols: high affinity, hatched symbols: intermediate affinity, white symbols: low affinity binding apoB.

immunophenotypes: high, intermediate or low affinity binding. Family studies indicated that distribution of these immunophenotypes (allotypes) probably resulted from the co-dominant transmission of two alleles, one coding for high affinity binding apoB, the other for low affinity binding apoB. The correctness of this hypothesis was demonstrated in a 28-member Finnish kindred (Fig. 2) in which an absolute co-segregation was observed between the allotypes defined by antibody MB-19, and the Ag(c) and Ag(g) epitopes already known to be transmitted in a co-dominant fashion (See below).

Antibody MB-19 Is an Anti-Ag(c) Antibody

Despite an initial negative report (8) the relationship between the Ag system and antibody MB-19-defined allotypes was re-explored. Simultaneous determination of complete Ag phenotypes and monoclonal antibody studies in Finnish subjects provided the necessary evidence. By coincidence, the first four-member family studied expressed an Ag phenotype pattern that was **identical with respect to all other Ag epitopes, but Ag(c) and Ag(g)** (Fig. 3). Figure 3 demonstrates that the Ag(c+g-) phenotype (=presence of Ag(c) only) coincided with high affinity binding, the Ag(c-g+) phenotype (=presence of Ag(g) only) coincided with low affinity binding. Moreover, the Ag(c+g+) phenotype (=presence of both epitopes) coincided with intermediate affinity binding. This key finding was found to be true in all other subjects studied (Fig. 2).

Thus the distribution of phenotypes can be explained by assuming two co-dominant alleles, one coding for apoB carrying the Ag(c) epitope (high affinity binding) and the other coding for apoB carrying the Ag(g) epitope (low affinity binding). These allelic apoB forms may be termed $apoB_c$ and $apoB_g$, respectively. In heterozygous subjects apoB carrying both Ag(c) and Ag(g) would be present resulting in the intermediate affinity binding pattern ($apoB_{c+g}$). This receives support from the in vitro studies of Young et al.(15) demonstrating that an equimolar mixture of high and low affinity binding apoB produced a Scatchard plot identical to that produced by preparation of intermediate affinity binding apoB, and located intermediate between the plots produced by high and low affinity binding apoB.

In summary, antibody MB-19 recognizes the the Ag(c) epitope. A double dose of the $apoB_c$ allele (c/c genotype) results in high affinity binding whereas a single dose (c/g genotype) results in intermediate affinity binding.

Distribution of ApoB Allotypes in Populations and Relation to Plasma Lipoproteins.

It is now possible to translate data obtained with antibody MB-19 into the "Ag language" and to compare such results with those obtained previously using human Ag antisera. For example, the least common allotype (high affinity binding =

	Ag phenotypes						anti-body
	x y	a_1 d	t z	h i	c g		MB-19
Mother	− +	− +	+ −	− +	+ −		high
Father	− +	− +	+ −	− +	− +		low
Child	− +	− +	+ −	− +	+ +		interm.
Child	− +	− +	+ −	− +	+ +		interm.

Figure 3. Identical Ag phenotypes except for Ag(c) and Ag(g) in a Finnish family (2) provided the key for translating antibody MB-19 binding patterns into the "Ag language".

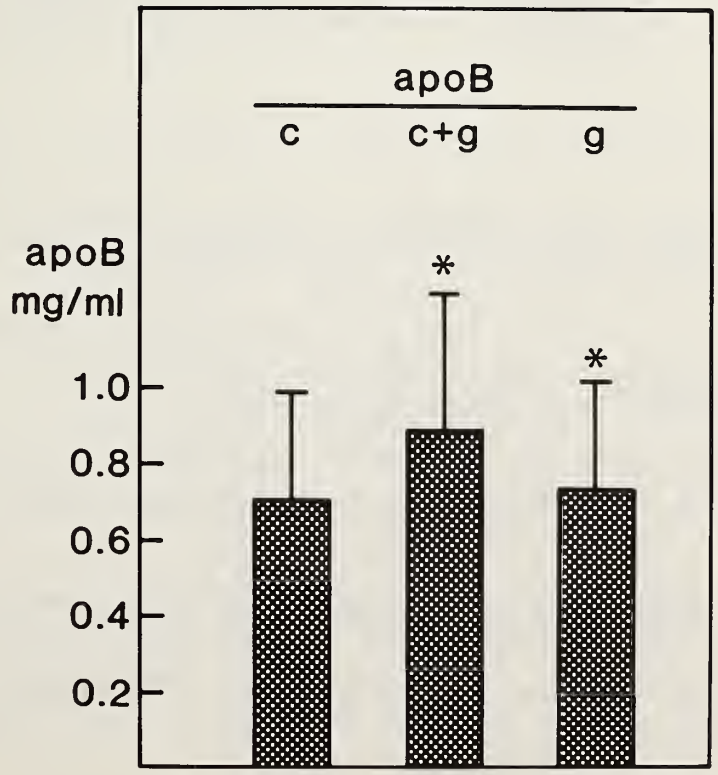

Figure 4. Concentration of LDL-apoB according to apoB allotype in young Finnish adults (n = 103).
* $p < 0.05$

Ag(c+g-)) determined by MB-19 assay occurred in 8.7% of Finnish subjects (16). Earlier assays with Ag antisera have shown this allotype to occur in 7.2% of Swiss (17), in 4.1% of Tibetan (18) and in 0% of Senegalese subjects (18).

Preliminary studies in Finland (2) and elsewhere (15) have indicated that LDL composition is the same regardless of apoB allotype as defined by antibody MB-19. However, at least in normal young Finnish adults the plasma concentration of apoB exhibited variation (Fig 4). The average levels of apoB in the two homozygous groups were similar, but the apoB in heterozygous subjects was significantly higher than in the low affinity binding ($apoB_g$) group. Studies in progress in Finland indicate that with increasing numbers of determinations the differences between the heterozygous and two homozygous groups become more apparent. The results suggest that homozygosity for the $apoB_c$ allele or the $apoB_g$ allele produce similar apoB levels in plasma. Only the heterozygous condition is associated with a relatively higher plasma apoB level. How the two alleles ($apoB_c$ and $apoB_g$), when present together, might interact to achieve this phenomenon warrants further study.

Acknowledgements

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LIPOPROTEIN ALLOTYPES CORRELATE WITH ATHEROSCLEROSIS AND HYPERCHOLESTEROLEMIA IN MUTANT PIGS

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Abstract

An immunogenetic survey of lipoprotein-borne allotypes was carried out in pigs. A model is presented which describes eight alleles of the pig apolipoprotein (apo)-B locus, Lpb, each apo-B variant consisting of eight allotypes (epitopes recognized by alloantisera). In addition to Lpb, two other lipoprotein-borne polymorphic antigens are described, Lpu and Lpr. Lpu is found exclusively in the LDL fraction of pig plasma, is genetically linked to Lpb, and might indeed represent another allotype on apo-B. Lpr is found in the $d<1.006$ g/ml and the $d>1.21$ g/ml fractions of pig plasma, is not linked to Lpb, and corresponds to a protein of 23,000 Mr. Two alleles have been found for Lpu and for Lpr, respectively. The Lpb5 allotype is associated with moderate hypercholesterolemia. In conjunction with the Lpu1 and Lpr1 allotypes, the Lpb5 allotype is associated with pronounced hypercholesterolemia and accelerated atherosclerosis.

Heritability of hyperlipidemia and lipoprotein polymorphism

Population genetic studies have conceded a heritability coefficient for hypercholesterolemia of approximately 0.5-0.6 (1,2). The frequency of hypercholesterolemia, approximately 0.1, is so high that even familial hypercholesterolemia, which results from the most common known mutations linked with hypercholesterolemia, (frequency=0.002), does not account for the majority of cases of hypercholesterolemia. For this reason, many investigators are actively searching for other genetic lesions which might be involved in occasioning the high prevalence of hypercholesterolemia.

In recent years dramatic strides were made in uncovering evidence of genetic variation in many of the apolipoproteins (3). Of particular clinical importance is the discovery that certain mutant alleles of apo-Al are common in individuals who have coronary heart disease and do not have hypercholesterolemia or any of the other classic risk factors for heart disease (4). This finding has set an important precedent because it establishes a new avenue by which a mutation in an apolipoprotein accelerates atherogenesis.

Progress in the discovery of apolipoprotein mutations leading to hypercholesterolemia has not been very swift. This is largely due to the fact that LDL is the principal cholesterol transporter in human plasma. Its predominant protein, apo-B, was for a long time intractable to analysis by conventional techniques of protein chemistry because of its unusual physical properties (high molecular weight, hydrophobicity, susceptibility to oxidation). Hopefully, this statement will soon be outdated because the human apo-B gene has been recently cloned in numerous laboratories. Identifying polymorphisms at the DNA level is a great deal simpler than dissecting the structure of this protein. Polymorphism in apo-B has instead been scrutinized with immunological probes (5), primarily monoclonal antibodies to defined epitopes. Although several monoclonal antibodies have identified polymorphisms in human apo-B (5), none of the polymorphisms have been correlated with any clinical phenotype.

Lipoprotein polymorphism in pigs

Eighteen years ago Rapacz embarked on a project whose chief aim was to identify polymorphic proteins in pig plasma. The studies were initiated by immunizing pigs with whole pig serum. The rationale behind this approach was the assumption that the most polymorphic proteins would be the most antigenic. If the animals were related, the selection for highly polymorphic proteins would be even more stringent. From the beginning, it was evident that strongest antibody responses were directed against lipoprotein-borne antigens. In our recent studies, in which the antisera were reacted with proteins electroblotted on nitrocellulose, we have shown unequivocally that the allotypes (epitopes recognized by the alloantisera) that were revealed by these alloimmune probes are part of the apo-B molecule. Thus, the remarkable degree of polymorphism associated with the lipoproteins is largely attributable to apo-B.

After screening a large number pigs and investigating the pattern of inheritance of various lipoprotein allotypes, Rapacz advanced an immunogenetic model for the polymorphism of beta-migrating lipoproteins (figure 1) (6). Sixteen apo-B allotypes were identified. The model describes eight apo-B alleles. Each allele encodes an apo-B containing eight of the sixteen allotypes. One of the eight allotypes is unique to to that allele. The remaining seven are shared by most of the other alleles. Thus, in figure 1, each double-digit numeral represents an allotype common to most of the alleles. Each single-digit numeral represents an allotype unique to that allele. The only exception is allele 8, which has the allotypes 5 and 8. Allele 8 might therefore be a mutation of allele 5, a "double mutant".

IMMUNOGENETIC MODEL OF Apo-B IN PIGS

Alleles	Epitopes (allotypic markers)
Lpb^1	[1] 12 13 14 15 16 17 18 21 22 23
Lpb^2	11 [2] 13 14 15 16 17 18 31 32 33
Lpb^3	11 12 [3] 14 15 16 17 18 31 22 23
Lpb^4	11 12 13 [4] 15 16 17 18 31 32 33
Lpb^5	11 12 13 14 [5] 16 17 18 31 32 33
Lpb^6	11 12 13 14 15 [6] 17 18 21 22 23
Lpb^7	11 12 13 14 15 16 [7] 18 31 32 33
Lpb^8	11 12 13 14 5 16 17 [8] 31 32 33

Figure 1. **Immunogenetic model for apo-B alleles in pigs.**

We have preliminary observations regarding the phenotypes corresponding to two apo-B alleles and more complete information regarding a third allele (described below). The $\underline{Lpb}^3$ allele is found in pigs which are significantly more resistant to the aortic fatty streaking that is induced by an atherogenic diet (7). Another apo-B allele, $\underline{Lpb}^7$, appears in animals with unusually low levels of plasma cholesterol. Recently, we discovered that LDL from $\underline{Lpb}^7$ pigs is able to stimulate cholesterol esterification in cultured mouse peritoneal macrophages at an unusually high rate (8). A small number of animals that were examined suffered from premature atherosclerosis. This mutation might therefore represent a unique model for studying atherosclerosis in the absence of hypercholesterolemia.

Hypercholesterolemia in Lpb^5 pigs

In his large survey of pigs with different apo-B alleles, Rapacz noted that some of the animals had hypercholesterolemia. Pigs with elevations in plasma cholesterol were all found to have the $\underline{Lpb}^5$ allele. These animals were found to be heterogeneous, however. Some had moderately elevated cholesterol levels (25% increase) while others had much more dramatically elevated plasma cholesterol (2-4-fold elevation). When each of these two groups of animals was immunized with the entire lipoprotein fraction from the other group, two additional lipoprotein-borne polymorphic markers were identified. The loci encoding these markers were named Lpu and Lpr (9).

Antisera against Lpu forms a precipitin reaction with LDL but not with the other lipoprotein fractions. Two alleles for Lpu have been identified, Lpu1 and Lpu2. Since this antiserum does not react with any protein on an immunoblot, we have not yet been successful in identifying the antigen. Lpu1 is only found in Lpb5 pigs. On the other hand, Lpu2 can be found in Lpb5 or non-Lpb5 pigs. Thus it is possible that Lpu is simply another allotype of apo-B and that the Lpb5 allele is more complex than the model shown in figure 1. There might be, for example, an Lpb5.1 and an Lpb5.2 allotype. Speaking against this model is the observation that antiserum to Lpb forms a separate precipitin line from antiserum to Lpu by double immunodiffusion (J. Rapacz, unpublished).

Antiserum to Lpr reacts on an immunoblot with a protein of 23,000 Mr. Two alleles were identified. Lprl corresponds to a protein with an isoelectric point of 6.8, although sometimes two forms are seen, 6.8 and 5.6. Lpr2 is a protein of isoelectric point, 5.4. Some Lpr2 animals also show a protein with an isoelectric point of 4.8. Heterozygotes always have the 5.4 and 6.8 forms and occasionally have all four. Treatment with neuraminidase does not change the number of bands on a two-dimensional gel (Checovich and Attie, unpublished).

The animals with the highest cholesterol levels have the genotype $\underline{Lpb}^{5/5}$ $\underline{Lpu}^{1/-}$ $\underline{Lpr}^{1/-}$ (where "-" can be allele 1 or allele 2). For the remainder of this paper, animals with this genotype will be referred to as "mutants". Due to a critical shortage of animals we have not been able to sort out the independent contributions of Lpu and Lpr to the lipoprotein phenotype.

The increase in cholesterol in the mutant pigs is predominantly in the LDL fraction ($p<0.005$). There is, however, a dramatic, 10-30-fold ($p<0.0001$) increase in IDL cholesterol. Since IDL in control pigs is normally very low (about 0.8 mg/dl), this large increase still makes only a minor contribution to the total cholesterol elevation (fig. 2). There is a moderate but significant ($p<0.01$) elevation in VLDL triglyceride (fig. 3).

Premature atherosclerosis in the mutant pigs

We examined the three main coronary arteries of 26 pigs of various lipoprotein genotypes. Seventeen were mutants ($\underline{Lpb}^{5/5}$, $\underline{Lpu}^{1/-}$, $\underline{Lpr}^{1/-}$) and 9 were controls ($\underline{Lpb}^{non-5}$, $\underline{Lpu}^{2/2}$). Both mutants and controls ranged in age from full-term fetus to 62 months. All pigs, after weaning, were maintained on a low-fat, 0% cholesterol diet. Non-$\underline{Lpb}^{5}$ animals (with the exception of $\underline{Lpb}^{7}$) did not show coronary lesions within the 5-year age span included in our study. Coronary lesions in pigs progress with age, typically beginning at eight years of age (10). In the control pigs, moderate lipid infiltration and medial hyperplasia were seen at 26 months. These features are considered normal in the absence of cellular changes associated with lesions. Neither lipid infiltration nor cellular changes were seen in older control animals.

Mutant pigs had moderate lesions in all three coronary arteries by seven months and severe lesions by 14 months of age. The lesions were characterized by intimal fibro-histiocytic cell deposition. Extensive deposition of cholesterol clefts at the junction between the intima and media, absence of fascicular architecture in the fibrous cap, and loss of lamellar units in the underlying media, were seen at 21 months. Cholesterol clefts, fibrous caps, and necrotic cores developed first in the distal portions of the right coronary and left anterior descending arteries. These features closely resembled those of human coronary atherosclerosis.

Normal LDL receptor activity in the mutant pigs

Fibroblasts were cultured from skin biopsies of control and mutant pigs. The rate of degradation of ^{125}I-LDL was measured in parallel cultures of each cell line. Both cell lines degraded ^{125}I-LDL at approximately the same rate, thus, unlike individuals with familial hypercholesterolemia or its animal counterpart, the WHHL rabbit (11), the mutant pigs have normal LDL receptor activity (12). It is therefore most likely that the mutations recognized by the alloantisera prepared by Rapacz (or mutations genetically-linked to these allotypes) are responsible for the hypercholesterolemia in these animals.

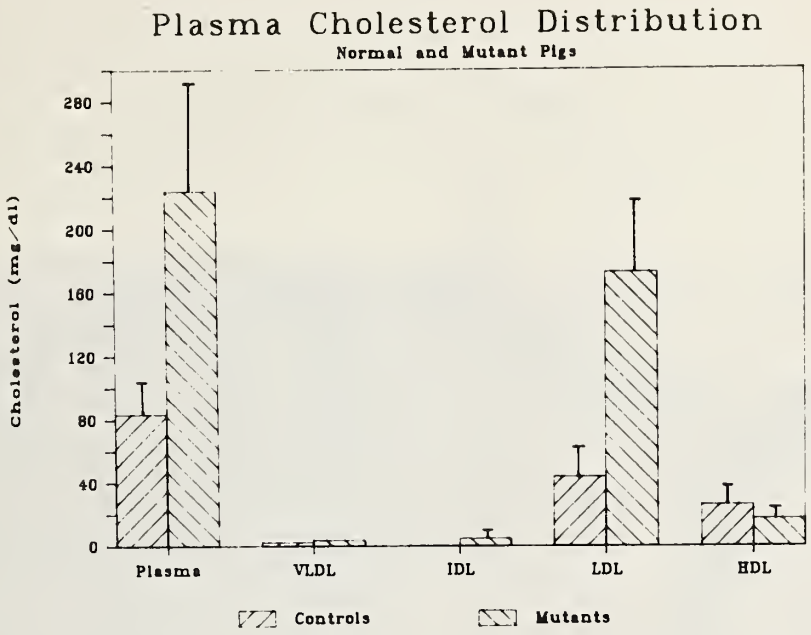

Figure 2. **Cholesterol distribution in the plasma of control and mutant pigs.** Bars represent means and standard deviations of determinations on 12 control and 8 mutant pigs.

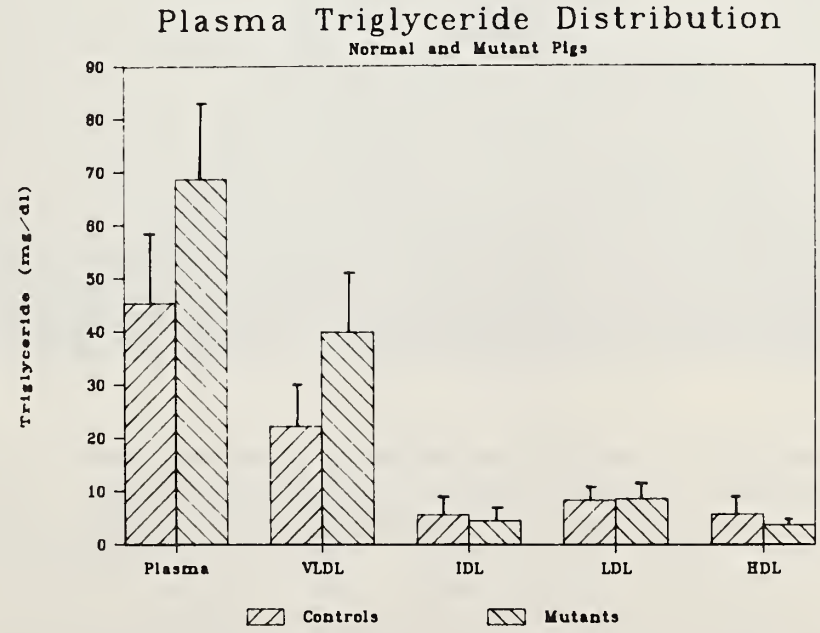

Figure 3. **Triglyceride distribution in the plasma of control and mutant pigs.** Bars represent means and standard deviations of determinations of 10 control and 6 mutant pigs.

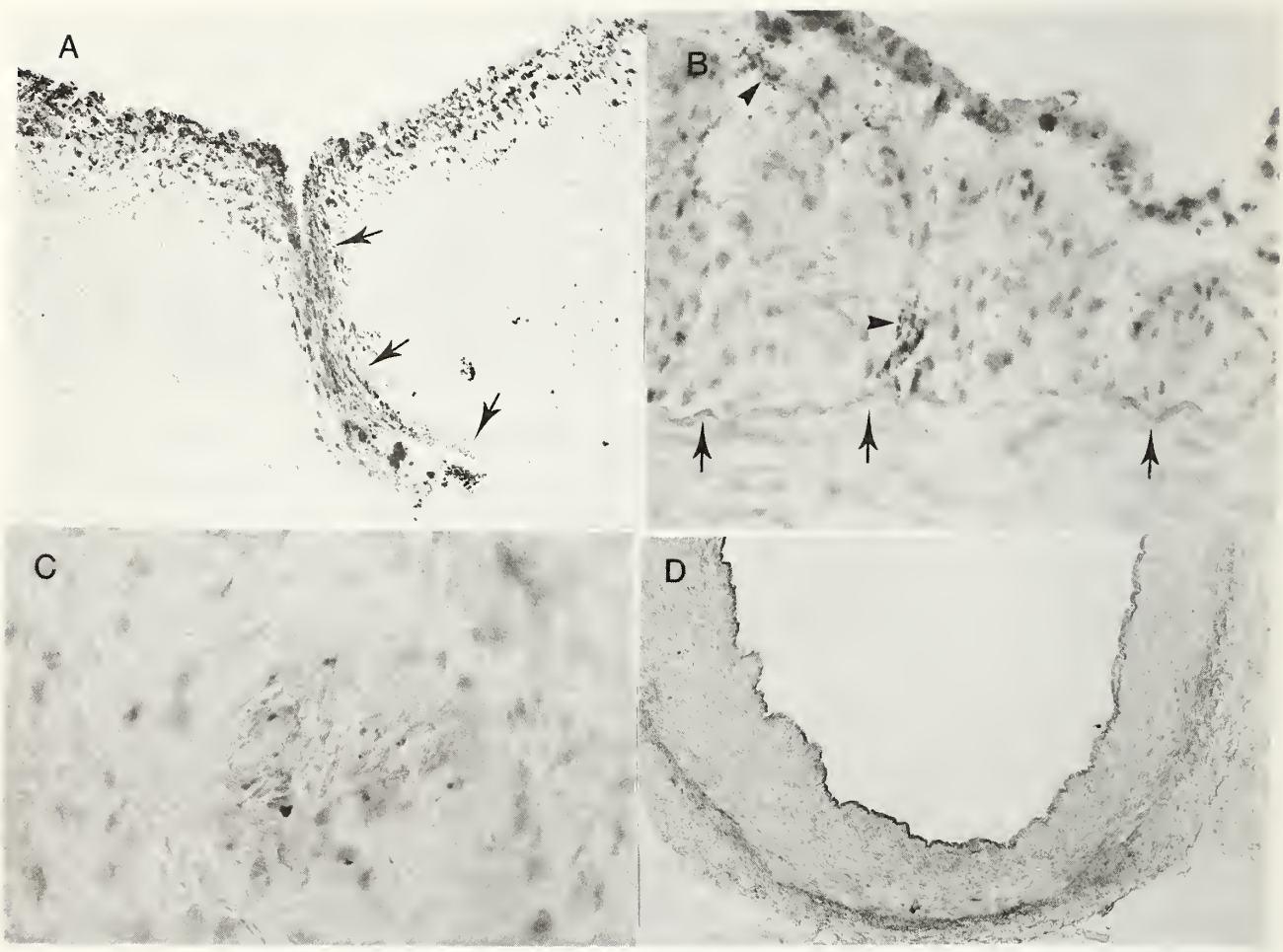

Figure 4. **Histological sections of coronary arteries from pigs fed a low-fat, 0% cholesterol diet.** A) Fatty streak lesions in the right coronary artery of a seven-month old pig with the genotype, Lpb$^{5/5}$, Lpu$^{1/1}$, Lpr$^{1/1}$. Lesions consist of fibro-histiocytic cells in the intima. Lipid-laden macrophages stain with Oil Red O. Foam cell or fatty streak lesions are usually found at branch sites (arrows). B) Higher magnification; internal elastic lamina (arrows) and macrophage foam cells (arrowhead). C) Right coronary artery of a 21-month old mutant pig. Cleft formations at the intimal-medial border, typical of extracellular cholesterol deposits in advanced lesions. D) Section from control pig, 14 months of age, stained for connective tissue. No edema, or architectural anomalies are evident. With lipid stain no deposition was seen (not shown).

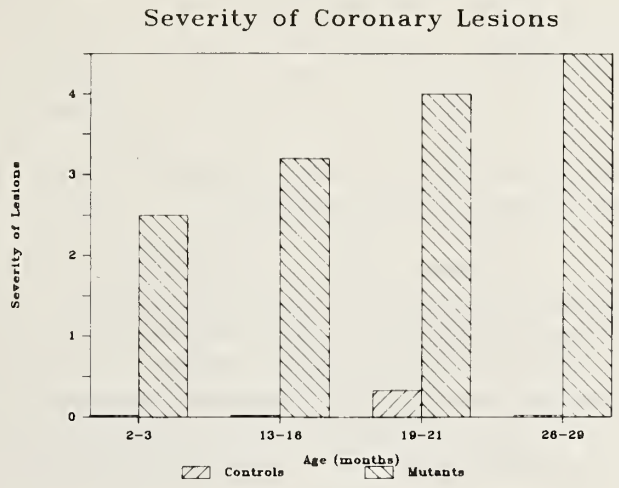

Figure 5. Relationship between age and severity of coronary lesions. Microscopic grading was performed on the three main coronary arteries of 26 pigs in the study. Mutant pigs were matched for age, sex, and heart weight. Coronary arteries were sampled at five standard sites and graded for: lipid infiltration, cellular hyperplasia, fibro-histiocytic intimal hyperplasia, lesion necrosis, formation of a fibrous cap, loss of fascicular organization, and atrophy in the underlying media. Grading reflects severity of lesions having two or more of the components listed.

Defective in vivo catabolism of mutant LDL

In vivo turnover studies were carried out in order to ascertain the mechanism underlying the increase in the concentration of LDL in the mutant pigs. Mutant and control LDLs (^{125}I- and ^{131}I-labeled, respectively) were simultaneously injected into normocholesterolemic non-Lpb^{5} pigs. The fractional catabolic rate of mutant LDL was about two-thirds that of control LDL. When a similar experiment was carried out in mutant pigs, the fractional catabolic rates were not significantly different, however they were about one-half of the fractional catabolic rate of control LDL in a control pig. It is therefore likely that the elevated LDL level in the mutant pigs leads to a down-regulation of the LDL receptor. This would decrease the fractional catabolic rate of LDL. Moreover, we can infer that the mutation in the Lpb^{575} LDL selectively impairs receptor-mediated LDL degradation in vivo because the mutant LDL is not cleared more slowly in the mutant pig.

The magnitude of the catabolic defect in mutant LDL catabolism parallels that of individuals heterozygous for mutations causing familial hypercholesterolemia. Familial hypercholesterolemic heterozygotes and our mutant pigs have a similar 2-4-fold elevation in LDL. Apparently the mutation in the pig is the "lock-and-key" companion of LDL receptor mutations insofar as the pig has a "key" mutation while LDL receptor mutations represent "lock" mutations.

Immunochemical studies suggest that human apo-B might be at least as polymorphic as pig apo-B. It is therefore conceivable that humans have apo-B mutations which are analogous to those of our mutant pigs. Given the high prevalence of hypercholesterolemia, such mutations might be quite common.

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SESSION VII. DISCUSSION OF GENETICS OF ApoB-CONTAINING LIPOPROTEINS

DR. GINSBERG: Dr. Brunzell, the simpler model for LDL kinetics, intravascular, extravascular equilibrations, single output was generated from the biphasic K curve. In your new model or your additional model, you still have that intravascular/extravascular equilibration. Does that mean that there are three exponentials on the curve or, if not, how much is that smaller pool? Are the rate constants much different?

DR. BRUNZELL: The main reason that Dr. Foster originally put the other pool in the plasma was due to the evidence for kinetic heterogeneity based on the U/P ratios, the urine to plasma ratios, of radioactivity because the slope of U/P ratio over the 14 to 21 days of the turnover was a very steep line, suggesting there was kinetic heterogeneity in the plasma compartment itself, assuming that everything actually comes back in. It was on that basis that it was added. This wasn't new to our work. It was actually your model that I presented here. It is Waldo Fisher's model.

DR. GINSBERG: Having two pools with different kinetics and different exits in the plasma, I'm not sure if there is a need to have an extravascular equilibration, other than the fact that we know that LDL is in the extravascular space.

DR. BRUNZELL: It is interesting. When you try to take the extravascular compartment off the traditional plasma pool, the data don't fit as well. When you try to put it on the other pathway, the data also don't fit. Dr. Foster couldn't make an extravascular compartment for this newer LDL plasma subspecies.

DR. FISHER: We initially formulated the more complex LDL pool that was derived having physically separated the LDL species, using a tritium label, and having to fit the data from two different LDL subcomponents which had distinctly different kinetic data. Under those circumstances we still needed an exchange compartment. I would justify the need for the exchange compartment in addition to the several components of these very heterogeneous hypertriglyceridemic LDL based upon fractionation of the LDL.

DR. KRAUSS: I wanted to make a comment related to the diet response that Dr. Brunzell showed in the latter part of the slides which relates to the previous session of this meeting as well and that is potential for genetic heterogeneity of the dietary response. We have characterized phenotypes or apparent phenotypes within the general population that are quite similar to the control and FCHL phenotypes that we've seen here and the FCHL phenotypes you called pattern B is actually much too common in the population. Approximately 15 or 20 percent of the population appears to have this LDL pattern with relatively normal although slightly elevated triglyceride levels. These are probably not true FCHL but they may form a pool in which FCHL is derived. At any rate, with that background, we have looked now at dietary effects in individuals with both the A and B forms and we found about three out of ten individuals with phenotype B whose LDL increased with polyunsaturated fat feeding in much the same way that you described in FCHL which perhaps gives some further homology to this phenotypic definition within the general population. It's not a common phenomenon, but it certainly is something to be aware of.

DR. BRUNZELL: If you do things to change VLDL, LDL sits there and doesn't do very much. But if you treat patients with familial combined hyperlipidemia, with a fibric acid derivative, and with weight loss and restabilize them the LDL paradoxically increase. It may say something about the distribution of the different LDL subspecies and where they come from.

DR. KRAUSS: In that first pathway I showed yesterday, it could represent simply a relative conversion from the small VLDL to LDL2; that pathway is then added on to the second pathway which is predominant in FCHL so that you have a double peak.

DR. BRUNZELL: Our data are entirely compatible. All of the data fit that.

DR. GRUNDY: There seems to be a high correlation between high turnover rate in these particles and their composition and I've often wondered if a particle has a high turnover rate if it doesn't have time to acquire the amount of cholesterol that it would if it circulated in plasma longer. So, if you treat these familial combined patients, with fibric acid or something that slows down their turnover rate, is that why they get larger? Maybe normal people have larger LDL because they circulate longer and acquire more cholesterol as they circulate?

DR. BRUNZELL: I suppose that is possible. The data we have would suggest, in fact, that what is happening is more related to what Dr. Krause mentioned, i.e. that when we do things to lower triglyceride levels more LDL comes in and it is material that previously only was only in VLDL and never got into the LDL density range and now is just a more buoyant subspecies, LDL2. So, I can't answer your question. It is an interesting speculation. I don't know.

DR. WITZTUM: The point was partially addressed by Dr. Grundy's question. I was going to also bring up the data of Eisenberg and Deckelbaum who have shown that in patients with hypertriglyceridemia maybe a couple of those had familial combined that if the LDL is isolated during a period of hypertriglyceridemia it has a decreased interaction with the LDL receptor intrinsically. If you then isolate it afterward there is an increase. One would predict that if one were to do that in vivo, assuming that nothing intravascular occurred, there'd be differences in rates.

DR. BRUNZELL: We've done the same kinds of studies. If we take LDL from somebody who has a narrow band dense LDL and somebody who has the other kind, we in fact do find differences. ironically, the differences seem to be related to the triglyceride content of the particle or in fact how much triglyceride was in VLDL and, independent of that, we unable to show any other abnormalities. So, we do find differences, but they all seem to be related to the triglyceride component of the composition of the particle and otherwise we don't see any differences.

DR. KUNITAKE: In your FCHL/LDLs, when you notice there is less lipid, is the density compensated for by the loss of non-B apoprotein?

DR. BRUNZELL: That's a very important question because there is something that doesn't look right there. If we look at the protein components of LDL across the whole density range there is E in the buoyant LDLs and there doesn't appear to be much in the more dense LDLs, but this difference is not enough to account for these apparent discrepancies in composition and density. Perhaps we're overestimating the total protein mass of the particle and maybe there is a non-epitope recognized part of ApoB that is actually missing. So, if we measured the mass by Lowry we might get a different answer, even though the total number of B moles is increased.

DR. GREGG: I would predict that the amount of transfer protein that is present in plasma is not going to be rate limiting. It's going to be like LCAT or ApoC2. There are going to be other factors that are going to be important and you've shown very nicely the different lines between controls and patients, that in fact there are different factors controlling that. Do you have data on enough patients? Is that due to the catabolism of the LDL? Is it due to different compositions in the VLDL populations in these patients versus controls which will help you come up with different lines?

DR. BRUNZELL: I don't know. There could be something wrong with the message for ApoB or maybe some post-translational abnormality, but we're hoping that it is something with the lipoprotein structure itself. I've taken lipoproteins from several different patients looking at this and there appears to be an abnormality in the LDL itself.

DR. WITZTUM: Dr. Sniderman, patients who have hyperapo-B small, dense LDL, but are normal triglyceridemic, how would you explain the etiology of their small LDL?

DR. SNIDERMAN: I don't think the static plasma triglyceride level is the sole or even the major explanation for the generation of the dense LDL. It comes to the number of particles that are being formed per unit time. The plasma triglyceride level is function of the input of VLDL particles and the hydrolytic capacity of the system. So that we see individuals who have small dense particles without elevated triglyceride levels. I have no doubt that dense LDL does occur in the frankly hypertriglyceridemic patients who are 500 or 1000mg/dl. But that is not the typical profile of hyperapoB certainly or for familial combined as well. The triglyceride levels are higher than patients without coronary artery disease, but it's the flux that counts. It's the number of particles per unit time that counts rather than the fasting triglyceride level.

DR. CHAIT: Dr. Sniderman, do you think that your patients who have familial hyperApoB in fact have the same genetic disorder as others with familial combined hyperlipidemia?

DR. SNIDERMAN: In a word, yes.

DR. GRUNDY: We've been very interested in hyperApoB and familial combined. The question that keeps coming up in my mind is how important is this in coronary heart disease? Dr. Joe Goldstein found familial combined in the Seattle group to be about ten percent of patients with coronary heart disease. There are some estimates that hypertriglyceridemia may occur in as much as 30 percent of patients with coronary heart disease. Can you just estimate? Is it a very common abnormality or so only maybe ten percent of patients have it?

DR. SNIDERMAN: As a clinical cardiologist, familial hypercholesterolemia is a rare disorder and does not account for five percent of the patients with coronary artery disease that we see.

In our hands hyperApoB is an extremely common disorder. The frequency could range from 30 to 60 percent, depending upon the way you slice your population, the age cuts and so forth. So, in our hands, it is very common. Hypercholesterolemia, per se, is not that common. No matter how the the LRC definitions change and the triglyceride issue is tougher to follow because they changed recently, but fundamentally it still runs five to ten percent and you expect five percent by chance alone. I'm not arguing that hypercholesterolemia is not a major risk factor for coronary artery

disease. I'm utterly convinced it is, but if our clinical encounters are typical and I don't have any reason to believe they are not, FH is not a major health problem compared to the overwhelming number of people that we see who have abnormalities of ApoB or HDL metabolism. It isn't even close.

DR. BRUNZELL: A comment to Dr. Grundy. What Dr. Goldstein said is under the age of 60, 14.7 percent had familial combined hyperlipidemia. The important thing is that this is a minimal estimate in his study because he had individuals who had heart attacks who had no relatives and he couldn't make a diagnosis. So, that number of 15 percent is the minimal estimate. I don't know if I would go as high as 60 percent, but it is a very common disease.

DR. SNIDERMAN: Can I just add that original definition demands a lipid abnormality and now in addition you have "normal lipidemic" high ApoB.

DR. BRUNZELL: Well, the normal values there, Dr. Sniderman, were for the probands and the 95th percentile for triglyceride was 165 at age 45. Now it's 300 and something. So, in fact, they were hyperlipidemic by different sets of criteria. It sounds as if you're saying the pathophysiologic defect in hyperApobeta familial combined is in fact an increased propensity for cholesteryl ester exchange.

DR. SNIDERMAN: No. It is increased hepatic ApoB synthesis and everything happens as a consequence of that. Too many ApoB particles being produced by the liver. There is something wrong with the regulation of ApoB synthesis in the liver such that, even at a reasonably early age, affected individuals synthesize too much ApoB and we found absolutely no evidence of a catabolic disorder. It all looks like over-synthesis and it seems to me the problem that we're preoccupied with now is to try to delineate whether the over-synthesis is due to an abnormality in the protein or whether it is due to faulty regulation of the protein. Undoubtedly, this is going to turn out to be an absolutely heterogeneous collection of disorders and it is important to emphasize this point because there must be about N number of points at which you can disturb the regulation of ApoB synthesis and all of them will produce the same monotonous kinetic defect. The kinetic results have by and large been monotonous in that they are ApoB over-synthesis.

DR. KRAUSS: My question is related because I'm trying to reconcile the description of hyperApoB and familial combined and the human type that we've reported in the normal population and one of the issues is the age of expression. We rarely find this disorder in children. There may be pockets, a subset of these genetic disorders which are indeed superimposed genetic trait. The children have the features, the blood protein features, similar or identical to those that you find in the adults or, if there are differences, what are they?

DR. SNIDERMAN: I'd emphasize the lack of data. I don't know of any good data for normal populations and the kinds of studies that we've done certainly have been targeted studies in which in the first instance we take patients who have coronary artery disease and hyperApoB and then look at their offspring, not looking at offspring in the total population. I just showed you a little bit of Dr. Kwiterovitch's data. Again that is a selected population, within which you'd expect it to be expressed, then it does come through. There must be variable ages of expression for a disorder that's likely to be a grab bag of disorders.

DR. KRAUSS: Are the HDLs low?

DR. SNIDERMAN: We haven't done the same fat load studies in the children, but it is much more frequent to see an adult with a low HDL than it is to see a child with a low HDL. There are very important metabolic changes as we age; obesity, etcetera that bring out the expression that our ability to clear lipoproteins is not as good as it used to be. Our hormonal interactions aren't the same as they used to be and some of the lipid, not to say endocrine functions, that that becomes evident as well. I'd argue that there is a need for a large amount of data within populations at risk and within the normal population to be able to really approach those questions.

DR. KRAUSS: Dr. Tikkanen, in the differences in the heterozygote and homozygote that you just showed and since the CG system is only one of five describing the AG system, one wonders whether a complex AG haplotype may exist in the population that affect the ApoB levels. This also could differ within different populations. Do you have any data on any of the other AG isoforms, allotypes?

DR. TIKKANEN: No, because as far as I know the antibodies that everybody is using don't detect any of the other and the supply of the AG antisera is very limited because only people who have received more than 40 blood transfusions--out of such people, a few percent will develop antibodies against LDL. So, that's why the studies have been very limited to just a few European centers.

DR. PACKARD: We know that between the ages of 20 and 40 one's LDL concentration sometimes doubles. Have you looked at older individuals to see if there is a change?

DR. TIKKANEN: I haven't seen any significant differences, in older subjects but I haven't looked systematically. I'm just analyzing all of the samples that get into the lab, but I haven't really clarified who is normal and who has coronary artery disease. But I know that this has been studied by Kwiterovich. He has a paper in press and he hasn't seen, as far as I have heard, any differences. Would you like to comment, Dr. Witztum?

DR. WITZTUM: There is also the paper of Steve Young. We've looked at 350 patients. We have three groups of patients. One group has about a hundred normal patients; a second group had 150 patients with coronary artery disease and a third group which consisted of patients with hypercholesterolemia. Within each individual group, we had identical frequencies of each of the three, very comparable to what Dr. Tikkanen's group reported. Also, again looking within each group, there was no difference in LDL cholesterol levels or any other lipoprotein measurement we could detect. So, we concluded that at least in adult southern California populations that--and I might point out also that we have done studies where we have isolated LDLs from individuals who are homozygote for either the high affinity or the low affinity and looked at their metabolism in cell culture, injected them into guinea pigs and other species and we have not been able to detect differences. We concluded, at least in southern California populations which are fairly homogeneous, that it wasn't associated with a metabolic difference. Of course, that might be different in other populations.

DR. PACKARD: Did you see a heterozygote effect?

DR. WITZTUM: No, not at all. For example within the coronary artery patients, the percent distribution of the three allotypes was identical. It was about 43-44 percent in the low reactors, etcetera. In other words, the distribution of the phenotypes were the same within each of the three groups and they were all identical.

DR. TIKKANEN: When you say southern California, you mean San Diego, because Los Angeles reports a difference.

DR. GINSBERG: If I'm correct, someone reported RFLP associations with the AG system at the Gordon conference. Could you clarify that information, and does it relate at all to this C antigen or is it a different antigen?

DR. TIKKANEN: Well, at the last Gordon conference, Vern Schumaker found that 14 of 17 subjects that he studied showed that the XBA1 RFLP was the same as the CG polymorphous. In 14 of 17 subjects he got the same results. He believes that they are very closely related.

DR. SNIDERMAN: Dr. Attie, I was with you to the point of your turnover curve. I didn't expect to see a catabolic problem, particularly given the cell data showing there was no difference in protein uptake.

DR. ATTIE: Oh, you probably mistook the cell data. The data were normal in mutant fibroblasts, not normal in mutant LDL.

DR. CHAIT: Have you done experiments using normal and mutant LDL on normal fibroblasts?

DR. ATTIE: We're doing that now. We've been delayed because we've had trouble thawing our pig cell lines. It has to be done in the pig cell lines.

DR. GINSBERG: Dr. Attie, have you injected mutant and control LDL into the mutants?

DR. ATTIE: Yes. What happens there is that they are not distinguishable and that the FCR in the mutant is about half of the FCR in the control. So, what is probably happening there is the high LDL levels are down-regulating the LDL receptor and therefore they can't discriminate the mutant LDL from the control LDL.

SPEAKER: Dr. Attie, do you have more detailed characterization of HDL and IDL on these pigs?

DR. ATTIE: No, we haven't done any more characterization of those particles.

DR. PACKARD: If you calculate the fractional catabolic rates, what do you get for a synthetic rate in the mutant?

DR. ATTIE: They are about the same.

DR. PACKARD: So, it's purely catabolic.

DR. ATTIE: That's right. That's why I said catabolism fully explains the elevation.

DR. ALBERS: I just want to make one comment about pigs. Pigs have a very little cholesterol esters transfer protein activity in plasma; however, they do have a significant amount of active transfer protein in the plasma compartment.

DR. HAVEL: Dr. Attie, it's been known for some time, as you indicate, that pigs have two different major LDL species, a peculiarity that nobody has previously addressed. Do you have any idea about the basis for that now related to the genetic studies and specifically are there any protein differences?

DR. ATTIE: We have no data on the protein differences.

DR. HAVEL: I mean other than ApoB.

DR. ATTIE: No. The best data on that are Dr. Goldsteins data. I think that there is heterogeneous metabolism of the heavy and light LDL fractions.

DR. WITZTUM: Isn't it true that in the pigs some of the earlier data showing that actually used density cuts of 1006?

DR. HAVEL: Yes.

DR. WITZTUM: So, what they were calling light LDL might be what we now--the same question we brought up before with Dr. Rudel. It really may not be quite analogous.

DR. SCHONFELD: Do you know where those mutations are in the gene, in the RNA?

DR. ATTIE: Which?

DR. SCHONFELD: The ones you were talking about, the fives and the sevens.

DR. ATTIE: That's where the major challenge of this project is going to be. There are quite a large number of polymorphisms.

DR. SCHONFELD: The implication is that there is something wrong with the way the proteins recognize the receptor. What region is it in? Would it be anywhere near where the people who are talking about the human receptor recognition site think the receptor site is?

DR. ATTIE: We suspect that that is probably somewhere in this exon here, but this is really very preliminary. We're nowhere near close to talking about that in detail.

DR. SCHONFELD: Can you explain about all of that lettering there?

DR. ATTIE: Those stars designate where we've seen differences in the comparisons of the different alelles and there are two kinds of differences that we've seen: site polymorphisms, meaning the presence or absence of a cut site and length polymorphisms, meaning that the gene is actually of a different size in that region and those are the two kinds of differences that we've seen and they are fairly large differences. But the major challenge is going to be to find which ones are relevant to the phenotypes of the animals. I think that most of these are going to turn out to be irrelevant.

DR. INNERARITY: Dr. Attie, could you give us any more information on ApoR? Is it really an apoprotein or is it something else?

DR. ATTIE: We don't really know very much about it. It is in the VLDL fraction and it is in the D greater than 1.21 fraction. Jan Rapacz just published a paper in "Genetics", a very extensive, thorough paper that describes the genetics of LP (R) and it's quite an impressive piece of work. He has also observed that there appears to be a distribution in ApoR after feeding; that normally the majority of this protein is in the D greater than 1.21 fraction, but after feeding a large amount of it appears in the VLDL or in the D less than 1006 fraction, but beyond that we really don't know much about it.

DR. KRAUSS: I want to make a remark regarding the question of the origins, the basis for the subspeciation of the different LDL populations in the pig. In addition to the studies that you showed, we've subfractionated the LDL in an equilibrium density gradient and taken fractions off the gradient and have data showing a rather remarkable homology of the five LDL components that we've seen on gradient gel with the size, density and patient characteristics of the LDL subspecies we found in humans. So, I wanted to make the point that the questions regarding LDL subspeciation in humans and pigs may have common answers.

SESSION VIII. High Density Lipoprotein

Chair: John Albers

ORIGIN AND HETEROGENEITY OF HDL SUBSPECIES

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Physical-Chemical Heterogeneity of HDL

Parameters describing the physical-chemical heterogeneity of HDL include hydrated density, particle size, apolipoprotein and lipid composition, and content of additional protein constituents (e.g., enzymes, such as lecithin: cholesterol acyltransferase, LCAT). Historically, the total HDL class has been defined according to its density of ultracentrifugal isolation, d 1.063-1.20 g/ml (1,2). Within this density interval two major subclasses, HDL_2 and HDL_3, have been identified with mean hydrated densities of 1.090 and 1.145 g/ml, respectively. More recently, additional ultracentrifugal subpopulations have been described within both the HDL_2 and HDL_3 subclasses (3,4).

By gradient gel electrophoresis (GGE), the particle size distribution of total HDL in normal subjects ranges from 12.9 to 7.2 nm and shows the presence, to a greater or lesser extent, of at least 5 major subpopulations (5). Based on total HDL particle size distributions determined for 194 human subjects by GGE (6), the ultracentrifugal HDL_2 subclass (12.9-8.8 nm) has been shown to contain 2 major size subpopulations, within size intervals designated $(HDL_{2b})_{gge}$ and $(HDL_{2a})_{gge}$, while the HDL_3 subclass (8.8-7.2 nm) contains 3 major size subpopulations, in size intervals $(HDL_{3a})_{gge}$, $(HDL_{3b})_{gge}$, and $(HDL_{3c})_{gge}$ (Fig. 1a).

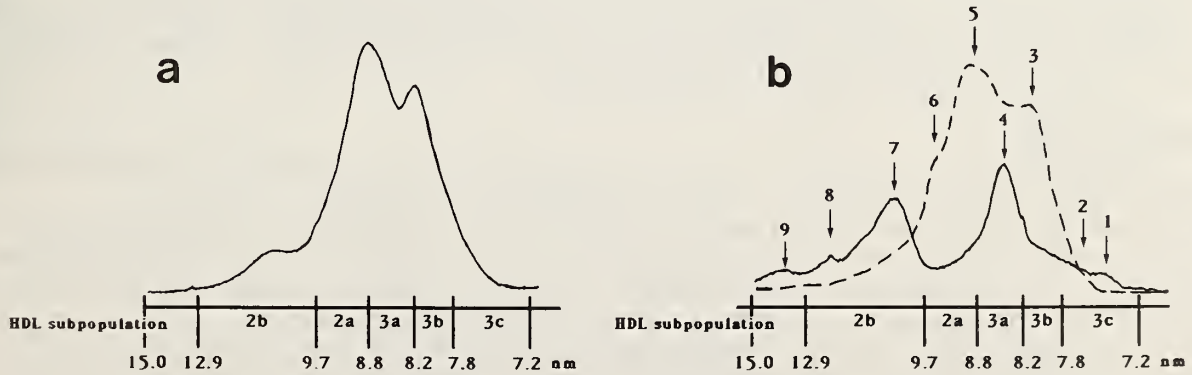

Figure 1: a) Representative GGE pattern of total HDL in a plasma ultracentrifugal d $\leq$ 1.20 g/ml fraction. Scale at base of pattern gives particle size intervals wherein peaks of major subpopulations occur in total HDL patterns of human subjects. The size intervals are designated: $(HDL_{2b})_{gge}$, $(HDL_{2a})_{gge}$, $(HDL_{3a})_{gge}$, $(HDL_{3b})_{gge}$, and $(HDL_{3c})_{gge}$. b) Representative GGE patterns of HDL populations separated from plasma by immunoaffinity chromatography (IAC). Solid line (———) designates pattern of HDL population containing apoA-I without apoA-II; dashed line (-----) designates HDL population containing apoA-I with apoA-II. Peaks are identified by numbers (at arrows) in a sequence proceeding from smallest to largest particle size for identifiable peaks in overlapping patterns.

Apolipoprotein Heterogeneity of HDL Particles

By IAC of human plasma, Cheung and Albers (7) have identified two major populations of lipoprotein particles whose protein moiety contains either both (apolipoprotein A-I (apoA-I) and apolipoprotein A-II (apoA-II)) or one (apoA-I) of the major HDL apolipoproteins. These populations contain particles that fall within the particle size range of the ultracentrifugal HDL. By GGE, the population with apoA-I but without apoA-II shows two major subpopulations in the HDL size range (Fig. 1b): one with mean size of 10.2±0.3 nm and the other 8.3±0.1 nm. These sizes fall within the size intervals defining, in total HDL, the $(HDL_{2b})_{gge}$ and $(HDL_{3a})_{gge}$ subpopulations, respectively. The population with apoA-I and apoA-II includes at least three major subpopulations with mean sizes 9.3±0.1, 8.8±0.1, and 7.9±0.1 nm, which fall within the size intervals of the $(HDL_{2a})_{gge}$, $(HDL_{3a})_{gge}$, and $(HDL_{3b})_{gge}$ subpopulations, respectively.

In addition to the major subpopulations occurring within the above two populations, minor components can also be observed in GGE patterns of the isolated populations. Such components are particularly apparent in the GGE pattern of the population containing apoA-I without apoA-II. The major and minor components, appearing as peaks and shoulders in GGE patterns of the two populations, can be designated by one sequence of numbers (1 through 9) which correspond to particle sizes (7.4 nm through 14.2 nm, respectively) (Fig. 1b). Segrest and co-workers (personal communication) have noted additional size heterogeneity within certain of the major subpopulations upon single vertical spin ultracentrifugation of plasma.

Based on the IAC studies, the major subpopulation (peak 7) that falls within the $(HDL_{2b})_{gge}$ size interval is apparently a species of HDL whose protein moiety includes apoA-I but no apoA-II. On the other hand, the major subpopulation (peak 3) that falls within the $(HDL_{3b})_{gge}$ size interval appears to be an HDL containing both apoA-I and apoA-II. Each population shows one subpopulation (peaks 4 and 5) within the $(HDL_{3a})_{gge}$ size interval. In the total HDL pattern, only one peak is usually observed in the $(HDL_{3a})_{gge}$ interval and it most likely represents a mixture of peak 4 and 5 subpopulations. Although present in lesser amount, other apolipoproteins (apoC, apoD, and apoE) are also detected in HDL populations and preliminary evidence indicates that their distribution is also heterogeneous (7).

Origins of HDL

Major processes involved in the origins of mature, core-containing HDL, as well as those contributing to their heterogeneity, appear to include: secretion by liver and intestine of diverse core-poor precursors containing apoA-I or apoE (both cofactors of LCAT); possible remodeling of such precursor species (e.g., by hepatic lipase (HTGL)); core formation within the precursors by LCAT, in the presence of substrate (unesterified cholesterol (UC) and phospholipid (PL)); and, lastly, remodeling of surface and core domains of the core-enriched products. Remodeling of core might entail the activity(s) of lipid transfer protein (LTP) (8), lipoprotein lipase (LPL) (8), HTGL (8), and LCAT; remodeling of surface might include surface remnant incorporation (9), apolipoprotein incorporation and/or interchange (10), and phospholipid content modulation via HTGL (11) and LCAT.

Potential, naturally occurring precursors to HDL have been observed in instances where LCAT activity is either low or not present. In human LCAT deficiency, the particle size distribution of putatively nascent HDL shows considerable heterogeneity both in size and shape (Fig. 2a) (12). Lipoprotein species encountered include large discoidal and small spherical particles (13). A comparably heterogeneous size distribution has been observed for apparent nascent HDL particles in conditioned medium of Hep G2 cells (Fig. 2b) (14).

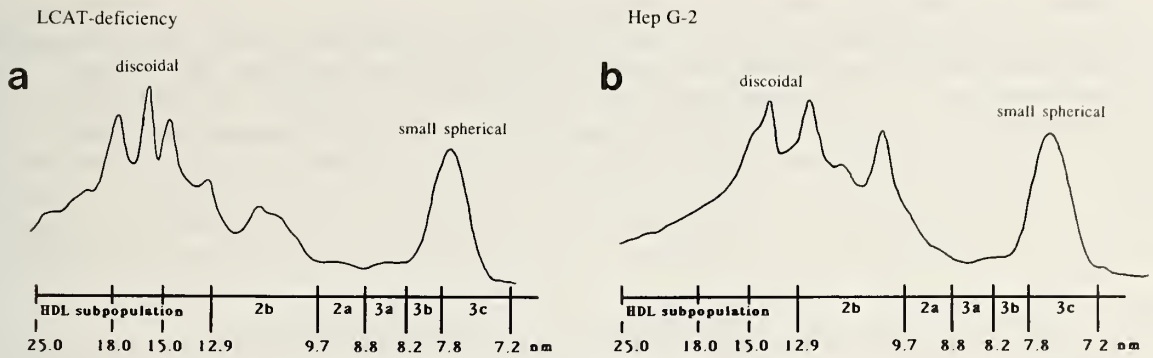

Figure 2: a) GGE pattern of apparent nascent HDL particles, migrating in the size interval between 25.0 and 7.2 nm, in plasma ultracentrifugal d $\leq$ 1.20 g/ml fraction isolated from plasma of patient with LCAT deficiency. Regions where particles of characteristic shape (discoidal or small spherical) occur are indicated. b) GGE pattern of apparent nascent HDL particles in ultracentrifugal d 1.063-1.20 g/ml fraction isolated from Hep G2-conditioned medium (14).

Discoidal and small spherical particles are present in the medium together with lipid-poor apoA-I. The major apolipoprotein associated with the small spherical particles in HDL of both LCAT deficiency and Hep G2 cell medium is apoA-I. ApoE and apoA-I are detected to a greater or lesser extent in the discoidal particles; the largest discoidal particles appear to contain predominantly apoE.

Nascent HDL from liver perfusate of African green monkeys show a heterogeneous size distribution containing both discoidal and spherical particles (Fig. 3a) (15). ApoE appears predominantly in the discoidal particles, while apoA-I is the major apolipoprotein in the small particles. The size distribution of HDL from rat intestinal lymph also shows a peak corresponding to small spherical particles and a broad band that includes both discoidal and larger, spherical particles (Fig. 3b) (16). Nascent HDL from both human and nonhuman sources thus consist of two morphologic types of particles: discoidal and small spherical species.

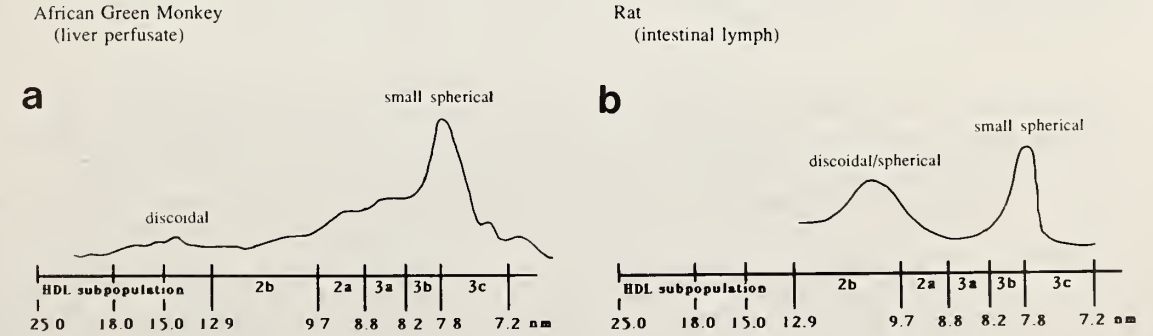

Figure 3: a) GGE pattern of apparent nascent HDL particles in ultracentrifugal d 1.006-1.225 g/ml fraction isolated from liver perfusate of African green monkey (15). b) GGE pattern adapted from a gel showing nascent HDL particles in ultracentrifugal d 1.13-1.18 g/ml fraction isolated from intestinal lymph of rat (16).

Information on the separate or coordinate contribution of the different nascent HDL to the formation of specific plasma HDL populations and subpopulation is rudimentary, due in major part to limited availability of native precursor material. To facilitate investigation of pathways of precursor transformation and origins of HDL subpopulations, we have prepared model analogs to nascent HDL whose properties closely approximate those of native precursor species (17,18). In this report we will consider pathways of transformation of two apoA-I-containing precursor analogs: (1) an analog to the small spherical HDL and (2) an analog to large discoidal precursor particles.

Analog to Small Spherical Nascent HDL and its Transformation

An analog to small spherical HDL particles (7.4-7.8 nm diameter) can be prepared by incubating discoidal complexes, containing 2 apoA-I per particle and egg yolk phosphatidylcholine (EYPC) in molar ratio of 95:1 (EYPC:apoA-I), with an LCAT-active plasma ultracentrifugal d > 1.21 g/ml fraction (18). The analog particle (7.8 nm diameter) exhibits a chemical composition (Table 1) very similar to that of the small nascent HDL in the liver perfusate of the monkey (15). The small nascent HDL in LCAT deficiency and intestinal lymph have a higher percentage content of protein.

Table 1: Composition of Small Nascent HDL and Analog Particles

Source	Composition (% wt)*					
	Pr	PL	UC	CE	TG	(FA)
Plasma (LCAT Deficiency)	60.3	34.6	2.9	1.2	1.0	
Lymph (Rat Intestine)	61	29	2	7	-	(1)
Liver Perfusate (African Green Monkey)	44.8	46.4	3.7	3.4	1.8	
Analog Particles	47.1	51.4	-	1.5	-	

* In this and the subsequent Table the abbreviations are: Pr (protein); PL (phospholipid); UC (unesterified cholesterol); CE (cholesteryl ester); TG (triglyceride); FA (fatty acid).

Incubation of the small particle analog with LCAT and a source of UC results in a quantum-step transformation to a larger, core-containing product in the size interval of the $(HDL_{3a})_{gge}$ subpopulation (Fig. 4) (18). The electrophoretic migration position and chemical composition of the product peak is similar to that of the smaller of the two major peaks (peak 4) in the size distribution of the (apoA-I without apoA-II)-HDL population. Specifically, the composition (wt%) of the analog transformation product at 24 h is: 47.8 (Pr); 34.9 (PL); 0.6 (UC); and 16.7 (CE). Preliminary values for the (apoA-I without apoA-II)-HDL subpopulation in the $(HDL_{3a})_{gge}$ size interval isolated by IAC from plasma are: 47.0 (Pr); 37.3 (PL); 1.3 (UC); and 14.4 (CE). A critical feature of this transformation is an increase in number of apoA-I molecules per particle from 2 apoA-I (in precursor) to 3 apoA-I (in product). This increase suggests a pathway involving fusion of two intermediates (each with 2 apoA-I) during core formation, accompanied by release of one apoA-I molecule from the product surface. This transformation is similar to that observed when small

spherical HDL (also with 2 apoA-I) from LCAT-deficient subjects are used as LCAT substrate in the presence of additional UC (19). The small HDL particles, both native and analog, contain exceedingly small amounts of UC and obtain their UC from the surrounding milieu. The major source of the phosphatidylcholine, however, appears to be the particle itself.

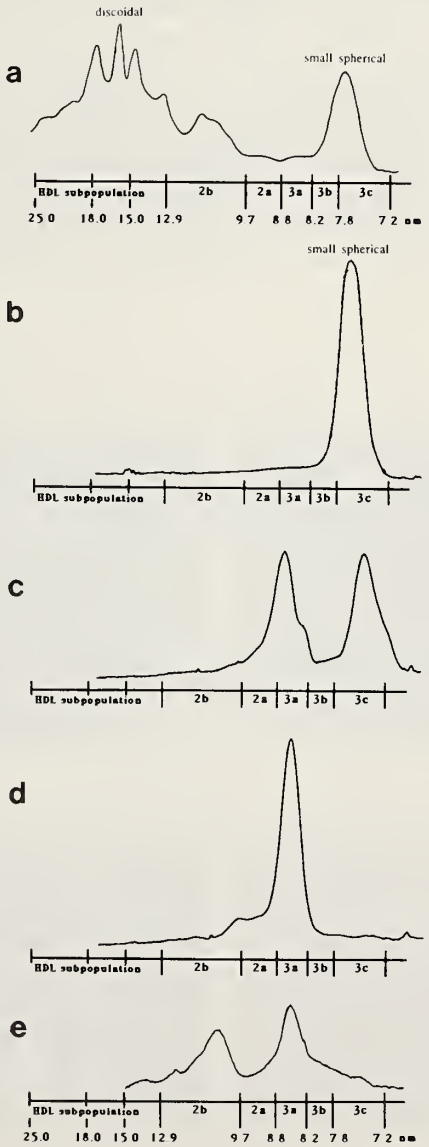

Figure 4: GGE pattern of: a) apparent nascent HDL isolated from plasma of subject with LCAT deficiency; b) analog to small spherical HDL; c) ultracentrifugal d 1.063-1.20 g/ml fraction isolated from incubation mixture at 6 h; mixture contained LCAT, source of UC (LDL), human serum albumin, and mercaptoethanol (18); d) ultracentrifugal d 1.063-1.20 g/ml fraction isolated from incubation mixture at 24 h; e) representative HDL population containing apoA-I without apoA-II isolated by IAC.

Transformation of the small HDL analog, in the presence of excess LCAT substrates, yields the same product observed at lower levels, suggesting an apparent saturation of core in the product particle with 3 apoA-I. Thus, under the conditions used, formation of a product corresponding to the other major and larger (apoA-I without apoA-II)-HDL subpopulation (peak 7), located in the $(HDL_{2b})_{gge}$ interval, does not occur when the small HDL analog with 2 apoA-I is used as precursor. This observation prompted us to consider the possibility that the larger HDL subpopulations in plasma may originate from precursors with a higher number of apolipoproteins per particle, such as the larger discoidal particles (15-20 nm, long dimension) observed in LCAT deficiency (20).

Analog to Large Discoidal Nascent HDL and its Transformation

Using the cholate method for apolipoprotein-lipid complex formation, we prepared discoidal particles with 4 apoA-I and long dimension 19.0 ± 3.0 nm (unpublished observations). LCAT-induced transformation of this large discoidal analog results in a product with particle size (12.5 nm, diameter) just within the upper bound (12.9 nm) of the $(HDL_{2b})_{gge}$ size interval (Fig. 5).

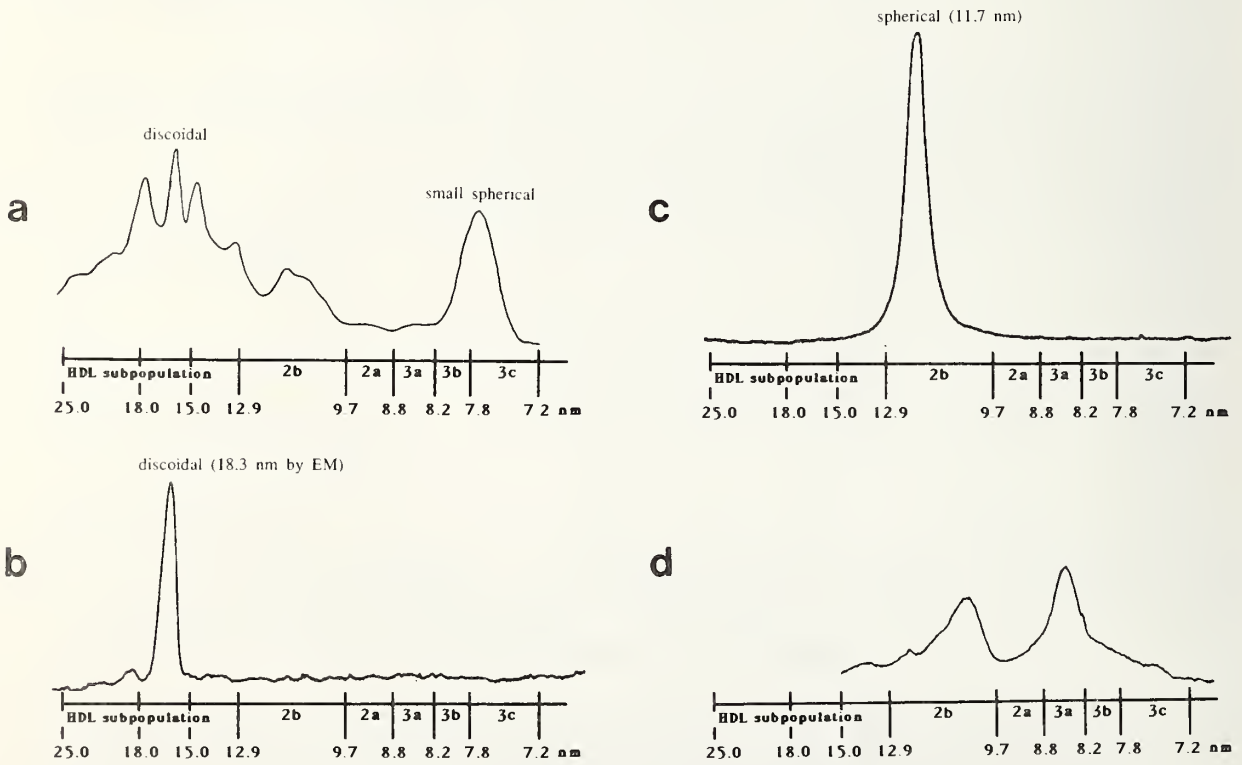

Figure 5: GGE pattern of: a) apparent nascent HDL isolated from plasma of subject with LCAT deficiency; b) analog to large discoidal nascent HDL prior to transformation in incubation mixture as described in legend of Figure 4c. Discoidal particles, with long dimension (by electron microscopy, EM) equal to diameter of spherical particles, migrate further on GGE than the spherical particles; thus, on the spherical diameter scale in this Figure, discoidal particles would be assigned a smaller size than actually measured by electron microscopy; c) ultracentrifugal d 1.063-1.20 g/ml fraction isolated from incubation mixture at 24 h; d) representative HDL population containing apoA-I without apoA-II isolated by IAC.

This region of particle size is characteristic of large HDL_2 particles that have been observed in abetalipoproteinemia (21). The transformation is characterized by an interesting progression of change in susceptibility to deformation upon negative-stain electron microscopy (Fig. 6). The precursor particles are discoidal with long x short dimensions of 19.0 x 4.4 nm. At 2.5 and 6 h, the particles appear rectangular with dimensions 14.5 x 7.5 nm and 12.0 x 8.1 nm, respectively. After 24 h, the transformation product is round and the particle diameter is comparable to that obtained by GGE (11.7 nm) when calibrated for globular proteins. Rectangular or square particles of similar dimensions have been observed in human lymph and shown to contain apoA-I and apoE (23). Our present studies suggest that such particles in lymph may be intermediate transformation products of large discoidal species. In view of our composition data on the intermediate transformation products of the analog (Table 2), the deformable lymph particles are probably also characterized by a comparably high phospholipid:cholesteryl ester molar ratio. Further core build-up in these intermediate particles would reduce the imbalance between surface and core contents and lead ultimately to formation of round, less deformable product particles.

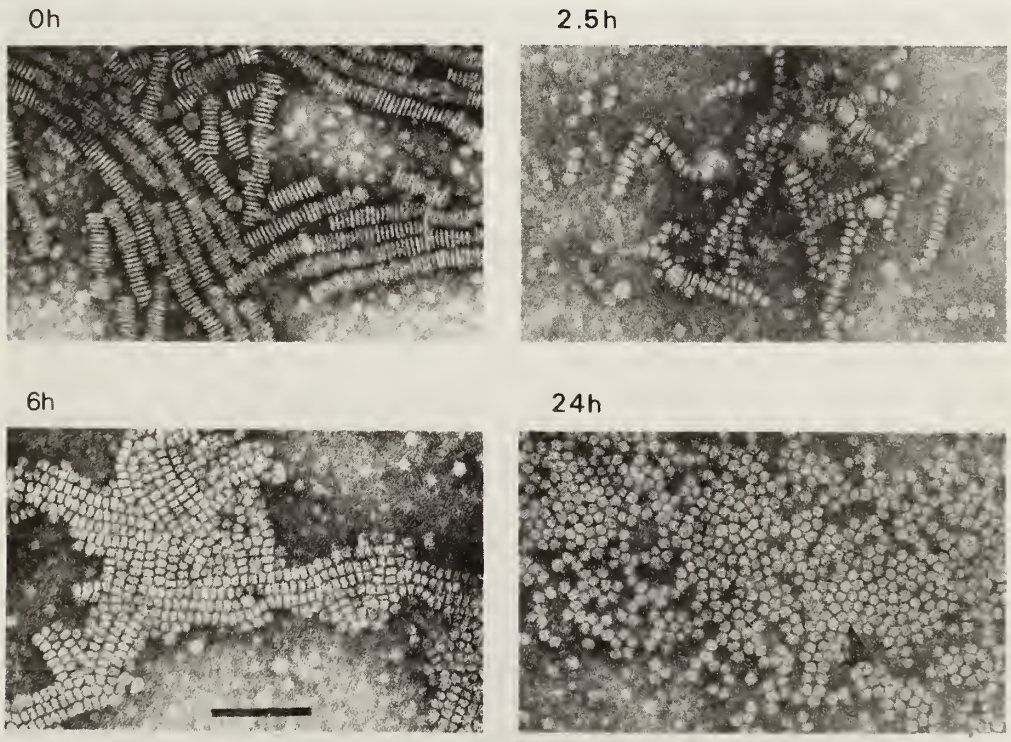

Figure 6: Electron micrographs showing time course of transformation of large discoidal analog. Samples were dialyzed to 5 mM ammonium bicarbonate (pH 8.0) and negatively stained with 1% sodium phosphotungstate (pH 7.4) (22). Bar marker on micrograph represents 100.0 nm.

Table 2: Composition of In Vitro Transformation Product of Large Discoidal Analog Particles

Sample	Composition (% wt)* Pr	PL	UC	CE
Large Discoidal Analog (0 h)	22.2	77.8	-	-
Product of Analog (2.5 h)	23.4	58.3	2.4	15.9 (120)**
Product of Analog (24 h)	23.0	49.1	1.5	26.4 (200)**

* Preliminary composition data for (apoA-I without apoA-II)-HDL subpopulation in $(HDL_{2b})_{gge}$ size interval: 38.8 (Pr); 28.7 (PL); 6.7 (UC); 25.8 (116)** (CE). Composition data for HDL_2 isolated by rate zonal ultracentrifugation (8): 37.7 (Pr); 31.3 (PL); 6.1 (UC); 20.7 (CE); 4.2 (TG).

** Values in parentheses are estimated number of core CE molecules.

The final product resulting from transformation of our discoidal analog is larger and different in composition from the predominant HDL_2 subpopulation in normal human plasma (Table 2). Values for the content of core lipid per molecule of the final product are also higher than estimated values for the major plasma HDL_2 subpopulation (4). If such large particles are formed in lymph or plasma, their further metabolism may involve either incorporation of apoE and clearance via the receptor-mediated pathway or a remodeling, by transfer of triglyceride for cholesteryl ester (by LTP) and lipolytic core reduction (by HTGL), to particles with properties of native HDL_2 subpopulations. It is altogether possible, however, that the discoidal analog we investigated may be larger than discoidal precursors which, in vivo, normally transform to HDL_2 subpopulations. Our more recent in vitro studies with smaller discoidal analogs are consistent with this hypothesis (unpublished observations).

Summary

Our studies with analogs to nascent HDL indicate that a major determinant of mature HDL particle size and apolar core content, in the absence of remodeling factors, is most likely the size and apolipoprotein content of the precursor particle. Depending on the number of apoA-I molecules per analog particle, the LCAT-induced transformation follows either a fusion pathway (for precursors with 2 apoA-I per particle) or a pathway (for precursors with more than 2 apoA-I per particle) that conserves the apolipoprotein number. According to our analog results, small nascent HDL probably serve as precursors to the major (apoA-I without apoA-II)-subpopulation in the $(HDL_{3a})_{gge}$ size interval. Our studies with the large discoidal analog suggest that HDL_2 (apoA-I without apoA-II)-subpopulations probably originate from the large discoidal nascent HDL that contain a higher number of apolipoprotein molecules per particle than the small nascent HDL. Intermediate transformation products of the large discoidal analog, described in the present study, resemble deformable species found in human lymph and are characterized by a relatively high surface-to-core lipid ratio. Whether large discoidal precursors containing apoE transform in comparable manner but with eventual interchange of apoA-I for apoE (10,15) is under investigation in our laboratory. Likewise, detailed delineation of pathways whereby the (apoA-I with apoA-II)-HDL subpopulations are formed is yet to be accomplished. Lastly, precursors relatively rich in phospholipid and initially poor in cholesterol would appear to be excellent candidates for reverse cholesterol transport by virtue of their

considerable capacity for uptake of cholesterol and LCAT-induced cholesteryl ester core formation.

Acknowledgements

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CHARACTERIZATION AND DISTRIBUTION OF HDL SUBPOPULATIONS

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Introduction

The high density lipoproteins (HDL) are conventionally defined as lipoproteins with densities between 1.063-1.21 g/ml. Their apparent bimodal distribution frequently observed in analytical ultracentrifugation has long been interpreted as two subclasses, HDL_2 and HDL_3. These two subclasses can be separated in a preparative ultracentrifuge using the density interval of 1.063-1.125 g/ml for HDL_2 and 1.125-1.21 g/ml for HDL_3(1). There is, however, evidence of considerable particle density and size heterogeneity within these two subclasses as investigated by equilibrium density gradient ultracentrifugation (2), zonal ultracentrifugation (3), and gradient polyacrylamide gel electrophoresis (4).

HDL particles also differ in their lipid and protein composition. HDL contains at least eight major apolipoproteins (apo)(A-I, A-II, A-IV, C-I, C-II, C-III, D and E), the enzyme lecithin cholesterol acyltransferase (LCAT), cholesterol ester transfer protein (CETP), and possibly an HDL conversion factor (5). These proteins are essential in maintaining the structural integrity of a lipoprotein particle. Many of them also play important roles in lipoprotein metabolism. LCAT and CETP are two key proteins postulated to be involved in reverse cholesterol transport (6). Apo A-I is believed to be the major physiological activator of LCAT (7). Apo A-IV, C-I, and E have also been shown to activate LCAT in vitro (8-12). Apo C-II is an activator of lipoprotein lipase (13). Apo E (14), and possibly Apo A-I and A-II (15-17) are involved in receptor-mediated lipoprotein catabolism. The recognition of the importance of these proteins in lipoprotein metabolism has led us to study HDL subpopulations based on their specific protein composition rather than their non-specific physical properties. The characteristics and distribution of some of these HDL subpopulations are described here.

Isolation of Lp(A-I with A-II) and Lp(A-I without A-II)

HDL is made up of two populations of particles which differ in their apo A constituents: particles that contain both A-I and A-II [Lp(A-I with A-II)]; and particles that contain A-I but no A-II [Lp(A-I w/o A-II)]. These particles can be isolated directly from plasma by using anti-A-I and anti-A-II immunosorbents (18,19). To isolate lipoproteins containing both A-I and A-II, plasma is absorbed with anti-A-II immunosorbent. The non-binding plasma proteins free of A-II eluted from the anti-A-II immunosorbent are absorbed with anti-A-I immunosorbent to isolate lipoprotein particles which contain A-I but not A-II. This procedure has been used in our laboratory to isolate these two populations of particles from the plasma of normolipidemic as well as hyperlipidemic subjects. In all subjects studied to date, substantial amounts of plasma A-I (25-55%) are found in particles that do not have A-II.

Lipid and apolipoprotein composition

Analyses of the lipid and protein contents of particles isolated from normolipidemic subjects show that on the average, Lp(A-I w/o A-II) particles contain slightly more lipid (55%) than Lp(A-I with A-II) particles (48%). Their lipid composition, however, appears to be comparable with the percentages of total cholesterol, phospholipid and triglyceride, being 33, 62 and 6, respectively. Irrespective of the original plasma A-I and A-II levels, immunochemical quantitation of A-I and A-II shows that the molar A-I/A-II ratio of Lp(A-I with A-II) particles isolated from 36 normolipidemic plasmas averages approximately 2.0 ($x \pm S.D.=1.89 \pm 0.29$). This suggests that in normolipidemic plasma, most Lp(A-I with A-II) particles contain two molecules of A-I and one molecule of A-II. However, the Lp(A-I with A-II) particles isolated from 16 hyperlipidemic plasma appear to have a slightly lower molar A-I/A-II ratio (1.59 ± 0.37). This ratio suggests that the Lp(A-I with A-II) populations of hyperlipidemic subjects may contain particles with A-I/A-II molar ratio of 2 as well as 1. Further studies using non-immunochemical methods are necessary to verify this observation.

Besides A-I and A-II, an average of approximately 60% of apo D and E in normolipidemic plasma are located in Lp(A-I with A-II) particles as determined by specific immunoassays. Lp(A-I w/o A-II) particles contain an average of about 30% of plasma apo D and 10% of plasma apo E. Analytical isoelectric focusing and urea or sodium dodecyl-sulfate polyacrylamide gel electrophoresis show that the apo C proteins are present in both Lp(A-I with A-II) and Lp(A-I w/o A-II) particles.

Distribution of LCAT and CETP in Lp(A-I with A-II) and Lp(A-I w/o A-II)

The distributions of LCAT and CETP in Lp(A-I with A-II) and Lp(A-I w/o A-II) have been studied in fasting normolipidemic subjects (20). Most plasma LCAT mass ($70 \pm 15\%$), LCAT ($69 \pm 16\%$) and CETP ($81 \pm 15\%$) activities are detected in Lp(A-I w/o A-II). Some LCAT mass ($16 \pm 7\%$), LCAT ($17 \pm 8\%$) and CETP ($7 \pm 8\%$) activities are detected in Lp(A-I with A-II). To determine the approximate molecular size of particles which contain these two activities, isolated Lp(A-I with A-II) and Lp(A-I w/o A-II) particles are further fractionated by gel filtration column chromatography. In Lp(A-I w/o A-II), most LCAT and CETP activities appear to be associated with different sized particles. LCAT activity is usually detected over a larger number of fractions than CETP activity. The mean hydrated Stokes diameters of particles containing LCAT and CETP activities are 11.6 ± 0.4 nm and 10.0 ± 0.6 nm, respectively. In Lp (A-I with A-II), most of the LCAT and CET activities are located in particles similar in size, 11.1 ± 0.4 nm and 10.6 ± 0.3 nm, respectively. Ultracentrifugation of a combination of Lp(A-I with A-II) and Lp(A-I w/o A-II) particles resulted in dissociation of both LCAT and CETP from these particles.

Size subpopulation of Lp(A-I with A-II) and Lp(A-I w/o A-II) in normolipidemic subjects

When Lp(A-I with A-II) and Lp(A-I w/o A-II) are separated by non-denaturing gradient gel electrophoresis and stained for proteins, distinct size subpopulations can be seen. Densitometric scanning of the stained lipoproteins show that Lp(A-I with A-II) particles of most normolipidemic subjects are made up of at least three size subpopulations. The mean hydrated Stokes diameters of particles in these three

subpopulations are 9.5 nm, 8.9 nm, and 8.0 nm, respectively. Particles with 8.9 nm diameter are usually the dominant subpopulation. In two of the subjects studied to date, the subpopulation with Stokes diameter of 9.5 nm appears to be missing (Fig. 1).

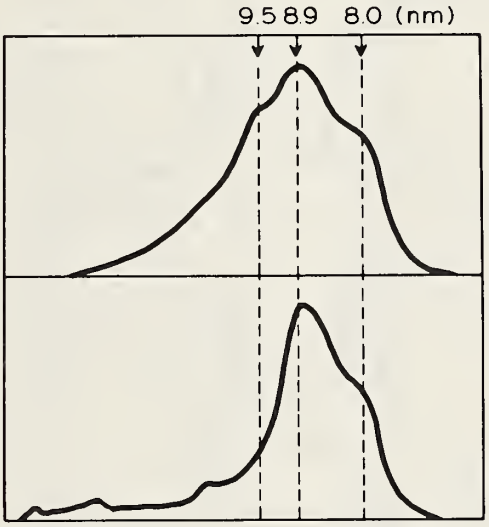

Fig 1. Densitometric scans of gradient gel electrophoresis of Lp (A-I with A-II) of normolipidemic subjects. Mean hydrated Stokes diameters of the three major subpopulations are indicated.

The Lp(A-I w/o A-II) particles of all normolipidemic subjects studied contain mostly particles of two discrete sizes with mean hydrated Stokes diameters of 8.5 nm and 10.5 nm, respectively. The proportion of these two size subpopulations varies widely among subjects, ranging from approximately equal proportion to a predominance of the 10.5 nm subpopulation (Fig. 2). Varying quantities of particles smaller than 8.0 nm and larger than 10.5 nm are often detected but are not found in every subject. Conventional HDL_2 and HDL_3 contains a mixture of Lp(A-I with A-II) and Lp(A-I w/o A-II).

Subpopulation distribution of Lp(A-I with A-II) and Lp(A-I w/o A-II) in coronary artery diseases (CAD) and hyperlipidemia

We have completed a study of the subpopulation distribution of Lp(A-I with A-II) and Lp(A-I w/o A-II) particles of patients with coronary artery diseases, both normolipidemic and hyperlipidemic. Irrespective of plasma lipid levels, the HDL subpopulations of CAD patients are different from healthy normolipidemic subjects. Compared to normolipidemic subjects with no symptoms of CAD, the Lp(A-I with A-II) particles of CAD patients contained an increased proportion of the 8.0 nm subpopulation as well as particles with Stokes diameter smaller than 8.0 nm. Furthermore, there is a decreased proportion of particles of 9.5 nm diameter (Fig. 3, left panel). Changes in subpopulation distribution of Lp(A-I w/o A-II) in CAD patients include: a) decrease of the subpopulation with Stokes diameter of 10.5 nm; b) increase of particles smaller than 8.0 nm; and c) increase of particles larger than the 10.5 nm subpopulation. The extent of these changes varies considerably among patients (Fig. 3, right panel).

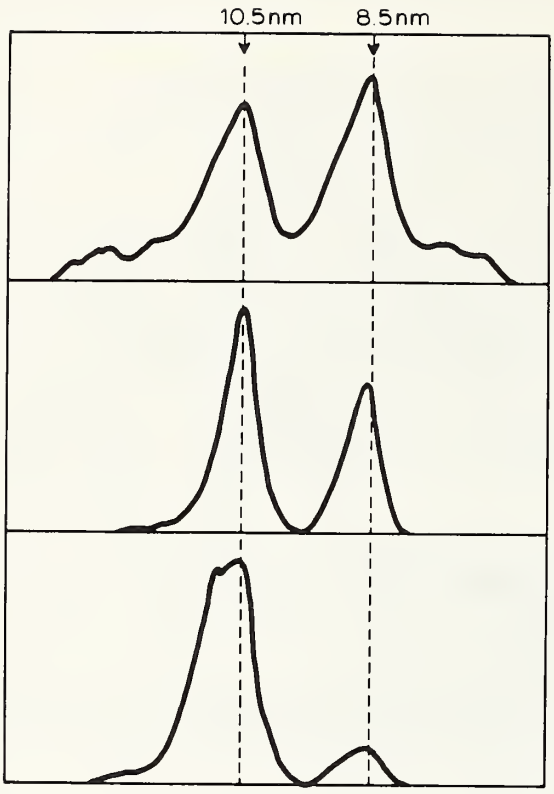

Figure 2. Densitometric scans of gradient gel electrophoresis of Lp(A-I without A-II) of normolipidemic subjects. Mean particle sizes of the two discrete subpopulations are indicated.

The observation of altered subpopulation distribution of Lp(A-I with A-II) and Lp(A-I w/o A-II) particles in both normolipidemic and hyperlipidemic CAD patients raises the question of the subpopulation pattern of subjects who are at risk for CAD, but are asymptomatic. To answer this question, we have begun to characterize the HDL of subjects with familial combined hyperlipidemia (FCHL). Although FCHL may occur in one out of 200 in the general population, perhaps one out of five subjects with premature CAD have FCHL. Our preliminary observation shows that subjects with FCHL also exhibit certain altered HDL subpopulation profiles similar to that of CAD patients i.e. increase of particles smaller than 8.0 nm and larger than 10.5 nm. An example of the subpopulation distribution profile of Lp(A-I with A-II) and Lp(A-I w/o A-II) of a subject with FCHL is shown in Fig. 4.

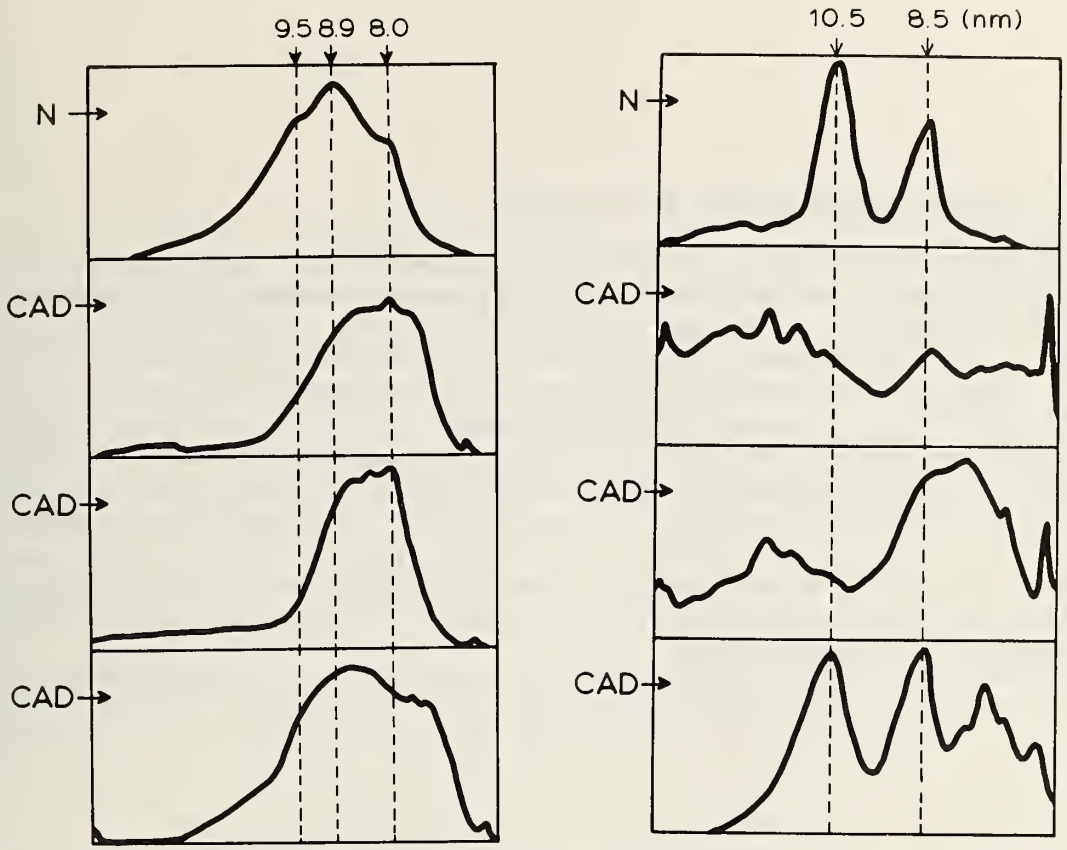

Figure 3. Densitometric scans of Lp(A-I with A-II)(left panes) and Lp(A-I without A-II) (right panel) of patients with CAD compared to healthy normolipidemic subject.

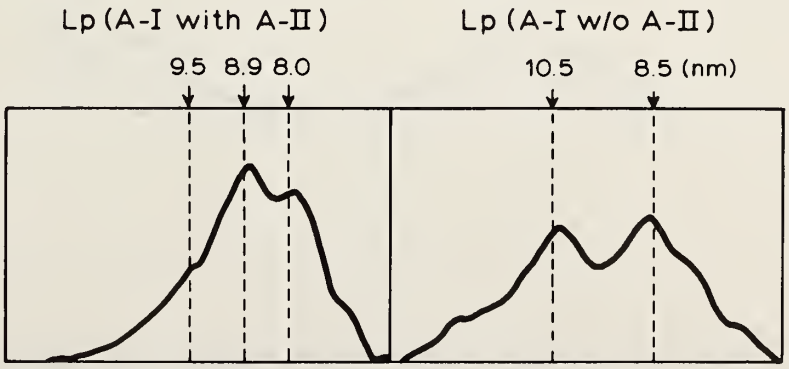

Figure 4. Densitometric scans of Lp(A-I with A-II)(left) and Lp(A-I without A-II) (right) of a subject with familial combined hyperlipidemia.

Effect of ultracentrifugation on HDL subpopulations

Ultracentrifugation is the most commonly used method for isolating lipoproteins. However, it has been shown that as much as 35% of plasma A-I can be disassociated from lipoprotein particles upon prolonged ultracentrifugation (21). This observation has prompted us to assess the effect of ultracentrifugation on HDL subpopulations. Lp(A-I with A-II) and Lp(A-I w/o A-II) particles are isolated either directly from plasma or from the d 1.063-1.21 g/ml fraction of the same plasma. Comparison of the subpopulation distribution shows that the Lp(A-I w/o A-II) population isolated from HDL has a reduced proportion of particles with hydrated Stokes diameter equal to or greater than 10.5 nm. This is true for particles isolated from both normolipidemic and hyperlipidemic plasma (Fig. 5). This suggests that in Lp(A-I w/o A-II),the larger-sized subpopulations are preferentially lost during ultracentrifugation. Based on similar densitometric scanning, little difference in subpopulation distribution is detected between Lp(A-I with A-II) particles isolated from plasma and HDL.

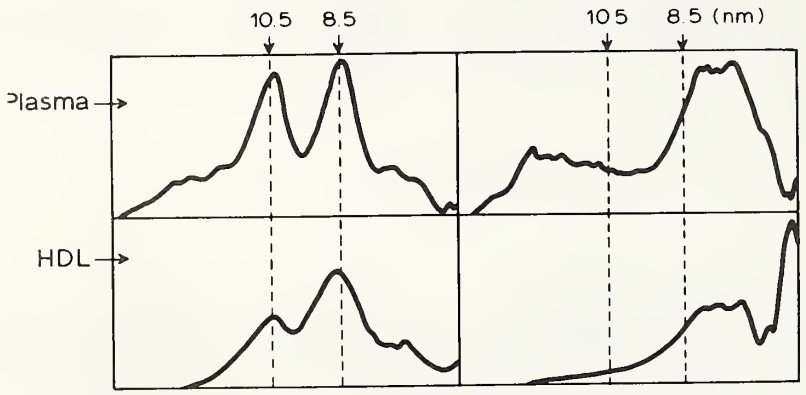

Figure 5. Densitometric scans of Lp(A-I without A-II) isolated from plasma (top row) and the d 1.063 - 1.21 g/ml fraction (bottom row) of a normolipidemic subject (left) and a hypertriglyceridemic subject (right).

Summary

HDL is made up of two populations of particles: Lp(A-I with A-II) and Lp(A-I w/o A-II). These lipoproteins can be isolated directly from plasma by immunoaffinity chromatography. Both populations of lipoproteins are heterogeneous in density and size. The distribution of the component size subpopulations are different between apparently healthy normolipidemic subjects and patients with coronary artery diseases or familial combined hyperlipidemia. Most plasma LCAT and CETP are associated with Lp(A-I w/o A-II). Sequential ultracentrifugation results in selective loss of LCAT and CETP as well as particles with hydrated Stokes diameters equal to or greater than 10.5 nm. Further studies are needed to elucidate the origin and metabolic significance of the various subpopulations.

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Abstract

HDL Subspecies: Structural Basis and Relationship to Atherosclerosis

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HDL subspecies structure. Analysis by nondenaturing gradient gel electrophoresis identified nine HDL subspecies, designated [1] through [9] in order of increasing size; [3-7] were the major subspecies. Lp(A-I with A-II) is composed primarily of subspecies [3],[5] and [6], and may contain some subspecies [2] and [7] while Lp(A-I without A-II) represents mainly [4] and [7] and the minor subspecies [1],[2],[8] and [9]. HDL subspecies [4],[5], and [6] are found in the standard sequential flotation density cuts for both HDL_3 and HDL_2, which illustrates the limitations of the latter terminology. Using single vertical spin ultracentrifugation, HDL fractions were isolated for physical and chemical analyses, including immunoassay for apo A-I, A-II, and C-II. An apo A-I-rich, lipid-poor subspecies was identified in the dense HDL fractions. HDL subspecies [7] was found to contain at least three separate subspecies designated [7a], [7b], and [7c]. Based on these results we propose that the HDL subspecies adjacent in size generally differ by the association/lack of association of a hinge-like domain of amphipathic helixes in a single molecule of apo A-I.

Lipoprotein subspecies, genetics and CAD. In a study of total VAP lipoprotein profiles obtained from 144 families with 11 year old monozygotic and dizygotic twins, analysis indicated that HDL_2 levels are highly genetically determined, with intrapair correlations of $r=0.81$ and $r=0.45$ for mono and dizygotic twins, respectively.

In two preliminary studies of the VAP lipoprotein profiles of white male patients with coronary artery disease (CAD) documented by cardiac catheterization compared with age and sex matched controls, HDL, HDL_3, and to a lesser extent, HDL_2 were strongly negatively correlated with CAD. No other lipoprotein classes were correlated with CAD.

HDL subspecies and CAD. The vertical autoprofile (VAP) procedure was used to study HDL subspecies distrbutions in a variety of subjects. Intrarotor coefficient of variation for the HDL-VAP curve shape was $3.5\pm 1.3\%$ for fresh plasma. Construction of a series of average HDL-VAP curves for plasma from normal male (n=35) and normal female (n=34) subjects indicated that HDL subspecies [3],[4],[5/6] and [7] can be identified in average curves.

Analyses of 20 male patients with documented 2 or 3 vessel coronary artery disease (CAD) revealed an average HDL-VAP curve similar to that of normal males in whom HDL[4] is the predominant subspecies. Difference analysis indicates that the average HDL-VAP from the CAD patients shows a marked decrease in HDL[5], HDL[6], and (to a lesser extent) HDL[7] when compared to the average male profile. In nine of the twenty CAD patients a prominant spike was seen in the dense HDL[2,3] region.

HDL Subspecies Structure

Introduction. Detailed studies of apolipoprotein compositional heterogeneity have been hampered by the lack of suitable methodology for isolation and quantization of even those HDL subspecies that differ only in size and density. Ultracentrifugation methodology that has been used for isolation of the different HDL subspecies (1-4) suffers particularly from extended centrifugation times (36 hr to almost 1 week) that lead to as yet incompletely characterized changes in the structural and compositional integrity of the isolated subspecies.

In view of the importance of the apolipoprotein components of an HDL particle in determining its functional role and metabolic fate, we have begun to isolate HDL particles with distinct apolipoprotein compositions using antibodies specific for each apolipoprotein (5). We found that apo A-I immunoaffinity chromatography removed virtually all HDL from plasma, indicating that most HDL particles contain apo A-I. On the contrary, apo A-II immunoaffinity chromatography removed only certain HDL density fractions from plasma. The present study reports the characterization of HDL subspecies using a combination of apo A-I and A-II immunoaffinity chromatography with single vertical spin (SVS) ultracentrifugation and negative stain electron microscopy. Ultracentrifugation by the SVS procedure has the advantage of short spin times (90-150 min) and thus less particle degradation than sequential flotation (6-8). Using this combination of techniques, we have identified a minimum of eleven distinct subspecies of HDL.

Inspection of multiple HDL-VAP curves indicated to us that individual HDL subspecies can often be identified by the recurrence of shoulders in the cholesterol profiles at reproducible positions. Thus, HDL-VAP procedure was used to analyze the HDL from a variety of plasma samples, including normal human male and female and males with documented coronary artery disease.

Vertical autoprofile analyses. The vertical rotor procedure (vertical autoprofle, VAP) used for the analysis of HDL subspecies was a modification of one described earlier for single spin separation and analysis of the major classes of lipoproteins (6-8). The gradient was formed in 5 ml Beckman Quik-Seal tubes (Beckman Spinco Division, Palo Alto, CA, cat. no. 34212) as follows: 1.3 ml of plasma was density adjusted to 1.21 g/ml and overlayered with 3.5 ml of a d = 1.06 g/ml KBr solution. The tubes were then placed in a Beckman VTi 80 vertical rotor and spun at 80,000 x g until $\omega^2 t = 3.00 \times 10^{11}$ (approx. 90 min).

The tubes are punctured and the effluent analyzed by a continuous flow cholesterol monitor developed in our laboratory (7). The basic system that allows computer sampling of the data has been described elsewhere (8).

Nondenaturing gradient polyacrylamide gel electrophoresis. The molecular size distribution of lipoprotein particles isolated from anti-A-I and anti-A-IIimmunosorbents was estimated by nondenaturing gradient polyacrylamide gel electrophoresis on Pharmacia precast PAA 4/30 gels and scanned densitometrically as described (5).

Isolation of HDL subspecies. For most of the HDL subspecies isolation studies, a rapid (2.5 hr.) single vertical spin (SVS) procedure was used to

isolate HDL subspecies fractions. Plasma samples (3.5 ml), adjusted to d = 1.18 g/ml with KBr, were overlayered with 13.5 ml of KBr solution at d = 1.12 g/ml and subjected to a 2.5 hr. ultracentrifugation in a TV-865B rotor at 65,000 RPM on a Beckman L8-80 or a Sorvall OTD-2 ultracentrifuge at 10°C. The tubes were fractionated by downward flow using a Hoeffer gradient fractionator and a Technicon or Pharamacia proportionating pump.

For one of the isolation studies, a 5.5 hr. SVS procedure was used to isolate HDL subspecies fractions. Plasma samples were adjusted to d = 1.21 g/ml with KBr and 5 ml was overlayered with 12 ml of a d = 1.06 g/ml KBr solution. This gradient was spun at 65,000 RPM for 5.5 hr. at 10°C in a TV865B Sorvall rotor and fractionated as described above. The collected fractions were analyzed for apolipoproteins and lipid.

Immunoaffinity chromatography. The most detailed analysis of the three HDL subpopulations eluted from the immunoaffinity columns, Lp(A-I), Lp(A-I with A-II), and Lp(A-I without A-II), is provided by nondenaturing gradient gel electrophoresis. We will use brackets to distinguish HDL subspecies defined by nondenaturing gradient gel electrophoresis (e.g. HDL[1], HDL[7a], etc.) from HDL subspecies defined by the analytical ultracentrifuge (HDL_3, HDL_2, etc.). In our terminology, the smaller, more dense HDL subspecies have the smaller numerical designators.

At least nine HDL subspecies based on size alone can be distinguished; five of these subspecies, [3], [4], [5], [6], and [7], represent the majority of total HDL. In the four subjects studied by immunoaffinity chromatography and nondenaturing gradient gel electrophoresis the Lp(A-I with A-II) was composed primarily of the major subspecies [3], [5], and [6] (there was also some [2] and [7]). Also, in all four subjects Lp(A-I without A-II) is composed primarily of the major subspecies [4] and [7], consistent with the bimodal nature of Lp(A-I without A-II), as well as the minor subspecies [1], [2], [8], and [9]. The mean Stokes diameters of the nine subspecies of these four subjects are shown in Table I. HDL_2 and HDL_3, isolated by the standard flotation density cuts (d = 1.063 - 1.125 and d = 1.125 - 1.21 g/ml, respectively) (4), overlap by several gradient gel particle sizes; HDL_2 contains [7] and [6], with some [5] and [4], whereas HDL_3 contains [3], [4], and [5], with some [6]. There is also some loss of resolution of the individual subspecies following the extended centrifugation.

Table I. Diameter of Individual HDL Subspecies Studied by Gradient Polyacryamide Gel Electrophoresis

HDL Subspecies	Mean Diameter in A
9	131.8 ± 1.1 (n=3)
8	121.4 ± 2.0 (n=2)
7	108.5 ± 3.8 (n=4)
6	95.3 ± 2.5 (n=4)
5	89.9 ± 2.4 (n=4)
4	85.0 ± 1.2 (n=4)
3	81.0 ± 0.9 (n=4)
2	78.2 ± 0.8 (n=4)
1	76.4 ± 0.8 (n=4)

Apolipoprotein compositional analyses of HDL subspecies. Plasma from one male and one female subject were subjected to the 2.5 hr. HDL-SVS procedure. Fractions were collected and analyzed for cholesterol and for apo A-II and C-II by radioimmunoassay (9,10). The results are consistent with the conclusion reached by the immunoaffinity chromatography studies just described, in that Lp(A-I with A-II), is complex and is located predominantly in the denser half of the HDL region. Subspecies [7], was evident by a flanking shoulder on the less dense part of the cholesterol curve. This is also the region of greatest C-II concentration, although other fractions also contain this protein. Further, the apo C-II and cholesterol curves showed three recognizable shoulders in the HDL[7] region, indicating that subspecies [7] is partially resolvable into three fractions. These three regions in the HDL[7] peak are designated as [7a], [7b] and [7c].

A comparison of the HDL-VAP profiles with the longer spin profiles correspond closely. More remarkable is the close correspondance between the HDL-VAP curves for Lp(A-I without A-II) and the curves for apo C-II. The correspondence between the HDL-VAP curves for Lp(A-I without A-II) and the curves for apo C-II raises the possibility that apo C-II may be able to substitute for apo A-II.

Isolation and analysis of HDL species. A third male subject was studied by whole plasma 2.5 hr HDL-SVS. Seven pools were identified based on analysis of the HDL-SVS curve and each pool was analyzed for apolipoproteins A-II and C-II by radioimmunoassay. These pools were also subjected to gradient gel electrophoresis. Since whole plasma was fractionated, plasma protein bands contaminate all of the pools, especially pools 1 and 2. Nevertheless, it is clear on the gradient gel that pools 5-7 contain the HDL subspecies [7]. Examination of the gradient gel analyses pools 5 and 6 suggests the presence of at least three different sized particles within subspecies [7]. Finally, these seven pools were subjected to HDL-VAP. The HDL-VAP results are qualitatively in good agreement with the gradient gel analysis. For example, the heterogeneity of HDL subspecies [7] was illustrated by both procedures.

Detailed compositional analyses of HDL subspecies. Plasma from a third female subject, was subjected to the 5.5 hr.r HDL-SVS fractionation procedure. A plot of three ratios, apo A-I/phospholipid, apo A-II/phospholipid and apo A-I/cholesteryl ester was compared. A significant feature of the three curves was a spike in the dense HDL region suggesting distinct subspecies in this region that are relatively enriched in apo A-I and apo A-II compared to both phospholipid and cholesteryl ester. The more dense half of this spike appears to be relatively apo A-II poor compared to the less dense half.

Results described here suggest that a new nomenclature for HDL subspecies is required. While we use both the broader designations Lp(A-I), Lp(A-I with A-II) and Lp(A-I without A-II) and the narrower designations HDL[1],[2],[3], [4],[5],[6],[7a],[7b],[7c],[8] and [9] for HDL subspecies in this paper, an ideal nomenclature should include apolipoprotein stoichiometry, as suggested previously by Alaupovic in his lipoprotein family hypothesis. In addition, it is possible that density and/or size will have to be considered in a future nomenclature to account for any HDL particle size variation due solely to apolipoprotein conformational differences. The results of the studies

reported here point to the significant limitations of the use of HDL_2 and HDL_3 fractions defined on the basis of standard sequential flotation density cuts of 1.063 - 1.125 and 1.125 - 1.21 g/ml, respectively, for studying HDL structure and metabolism.

The single vertical spin and vertical autoprofile procedures for fractionation of HDL have the advantage of extremely short spin times of 1.5 hr. for the HDL-VAP and 2.5 hr. for the HDL-SVS. Further, the HDL-VAP qualitatively reproduces the results obtained with immunoaffinity chromatography, gradient gel electrophoresis and electron microscopy.

We have arrived at HDL-VAP consensus positions for HDL[2],[3],[4],[5], [6],[7],[7a],[7b], and [7c], at 10 sec, 20 sec, 42 sec, 61 sec, 75 sec, 90 sec, 103 sec, and 120 sec, respectively. Time is translated from the tube position with the most dense being 10 sec and the less dense being 120 sec. Together, HDL-VAP, gradient gel analysis, and electron microscopy allow a tentative assignment of HDL subspecies positions to the preparative HDL-SVS fractionations. With the exception of HDL[7], assignment of more exact positions for the HDL subspecies must await isolation of subspecies [3-6] and HDL-SVS analysis on the analysis on the isolated peaks.

Table II is a compilation of physical and chemical data on the HDL subspecies [3],[4],[5],[6],[7a],[7b],[7c] and [8] and is based on the tentative assignment of positions for the subspecies on the HDL-SVS profiles. The apolipoprotein molar ratios represent averages of three to six separate analyses on a number of different subjects from three different laboratories using two different techniques (radioimmunoassay and radial immunodiffusion). It is apparent that as the diameter of the HDL subspecies increases, the molar ratio of total cholesterol to apo A-I increases, reaching a maximum of 83 in HDL[7c]. This increase parallels the corresponding increase in the volume to surface ratio for the HDL subspecies.

TABLE II. Summary of Physical and Chemical Properties of HDL Subspecies

HDL Subspecies	Diameter (Å)	Increase in Surface Area (Å 2)*	Average Increase Area (Å2)	Chol/A-I Molar Ratio	PL/A-I Molar Ratio	Apolipoprotein A-I Molar Ratio		
						A-II	C-II	D
3	81	----		13	5	0.52	0.01	0.06
4	85	2100		11	15	0.0	0.01	0.04
5	90	2700	2567	23	19	0.75	0.02	0.05
6	95	2900		29	30	0.95	0.05	0.07
7a	104	5627		33	57	0.71	0.09	0.12
7b	109	3346		38	72	0.44	0.15	0.19
7c	114	3502	3424	83	91	0.42	0.27	0.39
8	121	5900		--	--	--	----	--

*Over that of the preceding smaller subspecies. For example, a HDL-4 particle has a surface area 2100 Å 2 greater than a HDL-3 particle.

As shown in Table II, the difference in surface area of HDL[4] compared to HDL[3], HDL[5] compared to HDL[4], and HDL[6] compared to HDL[5] averages 2600 + 300 $Å^2$, the difference in surface area of HDL[7b] compared to HDL[7a] and HDL[7c] compared to HDL[7b] averages 3400 $Å^2$ ± 100 $Å^2$, while the difference in surface area of HDL[7a] compared to HDL[6] is 5600 $Å^2$. This is reminiscent of the results we previously noted for apolipoprotein A-I/DMPC recombinants (11), in which incremental changes in discoidal diameter between several discrete recombinant particles containing two apo A-I molecules per disc (R-2) on the one hand and between several discrete recombinant particles containing three apo A-I molecules per disc (R-3) on the other hand were quantized at ~ 13Å, while the difference between the diameters of the largest R-2 particle and the smallest R-3 particle was almost twice as large at 22Å. Our interpretation of these results was that the 13Å quantized changes in discoidal diameter represented quantized conformational changes in amphipathic helical regions of apo A-I, while the larger 22Å change represented the addition or subtraction of a third molecule of apo A-I. We would like to suggest here that similar mechanisms involving changes in apolipoprotein A-I conformation and stoichiometry may be involved in the structural, and probably functional, transitions between HDL subspecies.

We propose the following speculative model for the structure of HDL subspecies: (a) HDL subspecies [3],[4],[5] and [6] contain either two or three apo A-I molecules per particle and HDL subspecies [7a], [7b], and [7c] contain either three or four apo A-I molecules per particle. (b) The incremental changes in surface area between adjacent subspecies in the two different apo A-I stoichiometry classes are due to a single quantized conformation change in apo A-I and/or the addition of one molecule of apo A-II. (c) The single conformational change in apo A-I produces a hinge-like change, and thus a change in HDL surface-association, of a quantized number of amphipathic helical domains of apo A-I. (d) The apo A-I hinged domain is approximately equivalent in its effect on HDL surface area to that of a single apo A-II molecule. (e) The defect created by removal of the quantized unit of amphipathic helixes of apo A-I from the HDL surface, the hinged domain, can be filled by a single apo A-II molecule. (f) Apo C-II (and perhaps other apolipoproteins) can substitute for apo A-II.

Considering the possibility that there may be apolipoprotein heterogeneity within each of the HDL subspecies defined in this paper, the number of distinct HDL subspecies may be considerably greater than eleven. An important goal of our future research will be to further define the stoichiometry of HDL subspecies. It is hoped we can simultaneously establish a unifying concept to explain the structural complexity of these lipoproteins; the hinged-domain hypothesis is the initial working model. Structural knowledge should prove useful in attempting to understand the roles of the multitude of HDL particles in lipoprotein metabolism and atherogenesis.

Lipoprotein subspecies, genetics and coronary artery disease

Twin studies. In collaboration with the Departments of Human Genetics and Pediatrics of the Medical College of Virginia, a total of 300 families with 11 years old twins are being studied utilizing the VAP procedure for lipoprotein analysis. The results of total VAP profiles from 80 monozygotic and 64 dizygotic twins ahd their parents have been analyzed to date. Inspection

indicates that the concentrating pattern of HDL subspecies between monozygotic twins is highly conserved , as is the presence or absence of the variant lipoprotein Lp(a). Inheritance of high levels of Lp(a) and high or low levels of HDL from one or the other of the parents was often observed. The concentrations and patterns of LDL, IDL and VLDL were less well conserved than HDL and Lp(a) between monozygotic twins.

Formal analysis has shown that the levels of HDL_2 are dependent largely on inheritance; the intrapair correlactions between monozygotic and dizyaotic twins are r=0.81 and r=0.45, respectively. There was a significant correlation between the HDL_2 levels in the twins and the presence of a positive family history for coronary artery disease in the parents.

HDL Subspecies and CAD

Case-control coronary catheterization studies. Two separate groups of patients with documented coronary artery disease were studied. The first group were white male patients having catheterization at the UAB hospital whose plasma was sent for total VAP lipoprotein analysis by a physician in the Division of Cardiology. This group (n=35) is designated the referred cases. A second group of white males (n=41), designated the selected cases, were identified from the cath schedule on the basis of exclusionary criteria and plasma drawn before catheterization. Patients that were positive for renal disease, chronic liver disease, inadequately treated endocrinological diseases not including diabetes mellitus, cancer, congenital heart defects, heparin treatment, myocardial infarctions within the past 8 years and treatment with hypolipidemic drugs were excluded from the study. Following catheterization, the subjects were stratified into two groups: those having, and those not having, significant coronary artery disease (> 70°/o stenosis) in at least two of the three major branches. For all groups, age and sex matched controls were identified from a large group in the executive physical program at the UAB Diabetes Hospital who showed no evidence of CAD and on whom total VAP lipoprotein analysis had been performed.

The results of the case-control analyses (paired-t test) of the referred and selected cases showed HDL, HDL_3 ($P< 0.0001$ and < 0.0002; $P<.001$ and $P<.002$, respectively) being perhaps slightly more negatively correlated than HDL_2 ($P<.004$ and $<.006$, respectively). In the selected cases, VLDL cholesterol and total triglycerides were positively correlated with $P<0.06$. In neither group was LDL cholesterol or total cholesterol correlated significantly with CAD; the mean LDL and total cholesterol was slightly higher in the controls in both groups. Additionally, IDL was not found to be correlated with CAD.

Reproducibility of HDL-VAP. An intrarotor average plot generated by a computer from eight HDL-VAP analyses of fresh plasma aliquotes from a single female subject showed very little variability in the procedure. The mean intrarotor coefficient of variation of the HDL-VAP analyses on the eight fresh sample replicates is 3.5 ± 1.3°/o.

Average HDL-VAP curves for males and females. A collection of average HDL-VAP profiles of 35 normal young adult males were compared to average HDL-VAP profiles of 34 normal young adult females. These groups were subdivided into sets by predominate HDL subspecies present on the HDL-VAP. Some individuals were used in more than one subset. This analysis showed that

a prominant HDL[3] is more common among females (n=19) than males (n=6) and/or HDL[4] is lower relative to HDL[3] in females than it is in males. Further, when HDL[4] is prominant in males (but not when prominant in females) this pattern is associated with strikingly low HDL[5], HDL[6], and (to a lesser degree) HDL[7]. Finally, every one of the six types of average profiles in the males is associated with lower HDL[7] than is the corresponding average profile type in the females.

A difference plot of the average young adult male HDL-VAP curve subtracted from the average young adult female HDL-VAP curve was constructed. The most striking difference is an elevation in the female curve compared with the male curve between 65 and 140 sec, with a peak at approximately 110 sec. This represents the HDL[7a],[7b] and [7c] region. Two other smaller differences reflect slightly higher HDL[3] in females (elevation between 0 and 35 sec, peaking at approximately 20 sec) and slightly lower HDL[4] in females (peak centered at approximately 45-50 sec).

The most obvious difference between HDL-VAP analyses of male versus female human subjects is the significantly lower levels of HDL subspecies [7] in the males. HDL[7] is also the subspecies with the greatest variability in both sexes, except when the dominant subspecies is HDL[4], in which case HDL[4] shows the greatest variation. The HDL subspecies [7] is apparently identical to the fraction defined by nondenaturing gradient gel electrophoresis as HDL_{2b} (12), and is essentially synonymous with the fraction defined by zonal ultracentrifugation as HDL_2 (13). Thus the lower levels of HDL[7] observed in this study in males compared to females are to be expected (14-16). However, there is no precedence for the lower levels of HDL subspecies [3] and the higher levels of HDL subspecies [4] in males compared with females. One possibility is that HDL[4] substitutes in many males for some function expressed by HDL[7] in many females. Alternatively, the two subspecies may represent different steps in a sexually-influenced metabolic cascade.

Plasma HDL-VAP analyses of human males with documented coronary artery disease. A total of 41 white males undergoing coronary catheterization were selected for analysis by HDL-VAP. Of these, nine were found to have a prominant spike in the HDL[2,3] region of their HDL-VAP profiles.

Of the 41 white males selected for HDL-VAP analysis, 20 were documented as having significant coronary artry atherosclerosis in at least 2 vessels. A difference plot of the average HDL-VAP for the male controls subtracted from the average HDL-VAP for the males with documented coronary artery disease demonstrated differences. There are three regions of significant difference: 1) 0 to 50 sec. peaking between 10 to 20 sec (HDL[2] and [3]), that is highest in the coronary artery disease patients; 2) 50 to 120 sec. peaking betwee 60 and 80 sec (HDL[5],[6] and [7]), that is lowest in male controls; and 3) 120 to 160 sec. representing Lp(a), that is highest in coronary artery disease patients.

A noteworthy observation is that the average young adult male HDL-VAP profile prominant in HDL[4] is quite similar to the average HDL-VAP profile of the 20 males with documented coronary artery disease. Similarities include a prominant HDL[2],[3] and a prominant HDL[4] associated with a relative deficiency of HDL[5] and [6]. The prominant HDL[2], [3] region in the average

HDL-VAP profile for the males with coronary artery disease is at least partially due to the presence of a prominant spike in the HDL[2,3] region of the HDL-VAP profile of the 20 subjects.

The prominant HDL[2,3] spike in the subjects with coronary artery disease could be neither correlated with drug therapy nor with the presence of serum amyloid A as determined by radioimmunoassay in collaboration with Earl Benditt (results not shown). A speculative possibility is that this fraction is related to the protein (apo A-I)-rich, lipid-poor HDL fraction which has been reported elsewhere (17).

The results reported here demonstrate that the HDL-VAP has considerable utility for qualitative assessment of HDL subspecies levels in both humans and animals. The differences noted in the HDL subspecies patterns of males with documented coronary artery disease compared to male controls are intriguing and presently are under study. An effort to quantify HDL subspecies from the HDL-VAP curves by computer-assisted curve decomposition is the next step in the development of this methodology.

Regarding the role of HDL subspecies in atherogenesis documented 2 or 3 vessel coronary artery disease (CAD) revealed an average HDL-VAP curve similar to that of normal males in whom HDL[4] is the predominant subspecies. Difference analysis indicates that the average HDL-VAP from the CAD patients shows a marked decrease in HDL[5], HDL[6], and (to a lesser extent) HDL[7] when compared to the average male profile. In nine of the twenty CAD patients a prominant spike was seen in the dense HDL[2,3] region.

Conclusions

1. HDL is composed of at least eleven distinct subspecies.
2. The structural specificity for these eleven HDL subspecies partially resides in a hinge-like domain of amphipathic helixes in a single molecule of apolipoprotein A-I.
3. The pattern of HDL subspecies in a given individual is largely determined by genetic factors.
4. HDL and its subspecies are a more important risk factor for coronary artery disease in white males than the other lipoprotein classes and subspecies.
5. Coronary artery disease is most commonly associated with decreased concentrations of HDL [5], [6], and [7] and increased concentrations of HDL [2, 3] and [4] in white males.
6. This HDL subspecies risk pattern is similar or identical to the HDL [4] predominance pattern found in a minority of clinically-normal white males.

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IMMUNOCHEMICAL HETEROGENEITY OF HDL

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Abstract

Inherent heterogeneity of the expression of apo A-I epitopes on HDL has been described (Curtiss and Edgington, J. Biol. Chem. 260:2982, 1985). This heterogeneity was documented using three apo A-I-specific monoclonal antibodies and was characterized by the observation that none of the antibodies bound to and recognized all HDL in indirect, fluid-phase, immunoprecipitation radioimmunoassays. Here, we report our continued efforts to understand the basis for this immunochemical heterogeneity of HDL and document the generation of a new antibody that can bind all HDL. Deamidation of apo A-I in HDL can occur upon storage or alkaline treatment of HDL. Therefore, the effects of deamidation on the reactivity of seven apo A-I-specific antibodies were tested in competitive solid-phase radioimmunoassays. The three older antibodies, which were generated with stored apo A-I or HDL as the immunogen, had strikingly improved binding for alkaline-treated HDL, suggesting that deamidation of apo A-I could account for a large proportion of the incomplete reactivity of these antibodies. Furthermore, each of these three antibodies bound separate cyanogen bromide fragments of apo A-I, indicating that multiple determinants on apo A-I were sensitive to alkaline treatment. Four newer antibodies, which were generated using fresh HDL as the immunogen, had variable reactivity for alkaline-treated HDL. Among this group of new apo A-I-specific antibodies were antibodies whose reactivity was unchanged by alkaline treatment of HDL, as well as antibodies whose reactivity was either decreased or increased by alkaline treatment. More importantly, among this group of new antibodies was a single antibody whose binding was sensitive to alkaline treatment of HDL, that was capable of binding to and recognizing all radioiodinated HDL in an indirect fluid-phase immunoprecipitation assay. The results suggest that deamidation of apo A-I in HDL can have important implications for immunochemical reactivity of apo A-I, and that only some apo A-I monoclonal antibodies may be appropriate for use in measuring plasma levels of HDL.

Introduction

Using apoprotein (apo) A-I-specific monoclonal antibodies, intrinsic immunochemical heterogeneity of apo A-I was described (1). This heterogeneity was evidenced by the inability of any single apo A-I-specific antibody to bind or recognize all HDL in fluid-phase immunoprecipitation radioimmunoassays. In an attempt to explain the basis for this immunochemical heterogeneity, a number of technical issues were addressed. These

issues, including limiting amounts of antibody or antigens, radioiodination of the ligands, unavailability of the epitopes for reaction with antibody, selective binding to apo A-I isoforms, and individual allotypic differences in apo A-I, were not responsible for the incomplete antibody binding. To account for this apparent immunochemical heterogeneity of apo A-I, a number of additional hypotheses were examined. It was determined that the antibodies did not distinguish between apo A-I molecules from different synthetic sources such as hepatic versus intestinal apo A-I. Also, it was demonstrated that the antibodies did not distinguish absolute differences among HDL subpopulations that could be separated on the basis of size, density, or net particle charge. Therefore, no completely satisfactory explanation was found for the apparent inability of any single apo A-I-specific monoclonal antibody to bind all HDL (1). It should be pointed out, that in contrast to most apo B-specific monoclonal antibodies where it was demonstrated that they could bind and recognize all isolated LDL (2, 3), this incomplete binding was unique to the apo A-I-containing lipoproteins. Because such heterogeneity must be considered in an analysis of both the biology and physiology of HDL, we sought to understand the basis for this immunochemical heterogeneity.

Recently, Ghiselli et al. (4) reported their studies of the origin and the functional significance of apo A-I polymorphism in man. A number of isoforms of apo A-I can be distinguished in plasma by isoelectric focusing. These studies demonstrated that the major isoform, A-I_3 is the mature product of pro apo A-I or apo A-I_1 (4). Furthermore, they provided evidence that the other more acidic isoforms, namely A-I_4 and A-I_5 are derived from A-I_3 by a step-wise deamidation process, a process that occurs both *in vivo* and *in vitro*. At least some of the deamidation that occurs *in vivo* could be mimicked by alkaline treatment of the apoproteins *in vitro* (4).

Deamidation of apo A-I occurs upon treatment in alkaline solution. This non-enzymatic, hydrolytic deamidation, which occurs on asparagine and glutamine residues, has been observed in numerous proteins, hormones and synthetic peptides (4). Elevated temperature and pH facilitate deamidation and the rate depends upon specific buffers indicating a general base catalysis mechanism (5, 6, 7). We have therefore studied the effect of *in vitro* deamidation or alkaline treatment of HDL on the immunochemical reactivity of apo A-I.

Materials and Methods

Isolation of HDL and apo A-I. HDL was isolated from pooled normal human plasma by ultracentrifugation in the presence of protease inhibitors as described (1). Isolated HDL was dialyzed thoroughly against lipoprotein buffer containing 0.15 M NaCl, 0.3 M EDTA and 0.005% alpha tocopherol, pH 7.4 and was stored at 4°C. "Fresh" HDL refers to HDL that was used within a week of isolation.

HDL was delipidated with ethanol/ether and the apoproteins resuspended in 0.1 M sodium phosphate buffer, containing 0.1% sodium dodecyl sulfate (SDS). Apo A-I was isolated by high performance liquid chromatography (HPLC) with G3000SW TSK gel permeation columns as described by Kinoshita et al. (8). Fractions containing apo A-I were pooled and dialyzed against 0.01 M ammonium bicarbonate and stored at -70°C.

CNBr fragmentation was accomplished on lyophilized apo A-I dissolved in 90% formic acid (9). CNBr was added in a 13,000-fold molar excess and the reaction mixture incubated overnight at room temperature. Following lyophilization, the peptides were solubilized in 1% SDS, 0.01 M Tris, pH 8.2 and subjected to isoelectric focusing as described below.

All lipoprotein and apoprotein concentrations were estimated by a modification (10) of the method of Lowry using bovine albumin as a standard.

Apoprotein Electrophoresis. The apoprotein composition of the isolated lipoproteins was analyzed by SDS polyacrylamide slab gel electrophoresis as described (1). The running gels contained a linear 7.5% to 20% acrylamide gradient. The apo A-I isoforms were separated by isoelectric focusing in 6% polyacrylamide slab gels containing 8 M urea and 2% ampholine (pH 4 to 6) as described (1). Lipoproteins were delipidated by boiling for three minutes in 1% SDS before electrophoresis, and the gels were stained after electrophoresis with 0.1% Coomassie Brilliant Blue R250 in 50% trichloroacetic acid. Gels containing radioiodinated lipoproteins were visualized by autoradiography as described (1).

Hybridomas. The generation of the hybridomas secreting antibodies AI-4, AI-7 and AI-9 has been described (1). The four new monoclonal antibodies, AI-10, AI-11, AI-13 and AI-14, were obtained from three separate fusions of the spleens of Balb/c mice immunized as described (1) with either glutaraldehyde-fixed HDL or native HDL using standard fusion protocols as published previously (2, 3). The glutaraldehyde cross-linked HDL was prepared by exposing fresh HDL in phosphate buffered saline at 20°C for 18 hr to glutaraldehyde at a final concentration of 0.04%. Culture supernatants were screened for their capacity to bind both immobilized HDL in solid-phase radioimmunoassays (RIAs) and radioiodinated HDL in fluid-phase RIAs. Positive hybridomas were cloned at least twice by limiting dilution. Ascites fluids containing the antibodies were obtained from Balb/c mice which had been primed with 0.3 ml of mineral oil and injected intraperitoneally with 5×10^5 cloned hybridoma cells.

Immunoassays. Solid-phase RIAs were performed in polyvinyl chloride microtiter plates using the volumes and buffers described previously (1). The plates were coated with fresh native HDL and incubated with serial dilutions of mouse serum, hybridoma culture supernatants or ascites fluids for 18 hr at 4°C. Antibody binding was detected by a second 4 hr incubation with radioiodinated goat anti-mouse Ig. Competitive assays were performed by incubating limiting, fixed amounts of mouse monoclonal antibody simultaneously with the HDL competitors.

Fluid-phase immunoprecipitation RIAs were performed exactly as described (1). Precipitation of ^{125}I-HDL was accomplished with a slight antibody excess of goat anti-mouse Ig antiserum. Radioiodinated HDL was prepared as described to specific activities of 3 to 5 μCi/μg.

Western blot analyses of the apo A-I antibodies were performed as described previously (1-3). Electrophoretically separated proteins were transferred to nitrocellulose for reaction with dilutions of mouse monoclonal antibody. Monoclonal antibody binding was detected by a second

incubation with radioiodinated goat anti-mouse Ig followed by autoradiography.

Results

Isolated fresh HDL was incubated for 18 hr at 20°C in the absence or presence of increasing concentrations of 1 N NaOH. Following neutralization with 1 N HCl, the HDL was delipidated with absolute ethanol and anhydrous ether (1:1) and applied to a polyacrylamide isoelectric focusing gel (Figure 1). Visual assessment of the stained gels indicated that untreated HDL contained predominately the major isoforms A-I_3 and A-I_4 and minimal

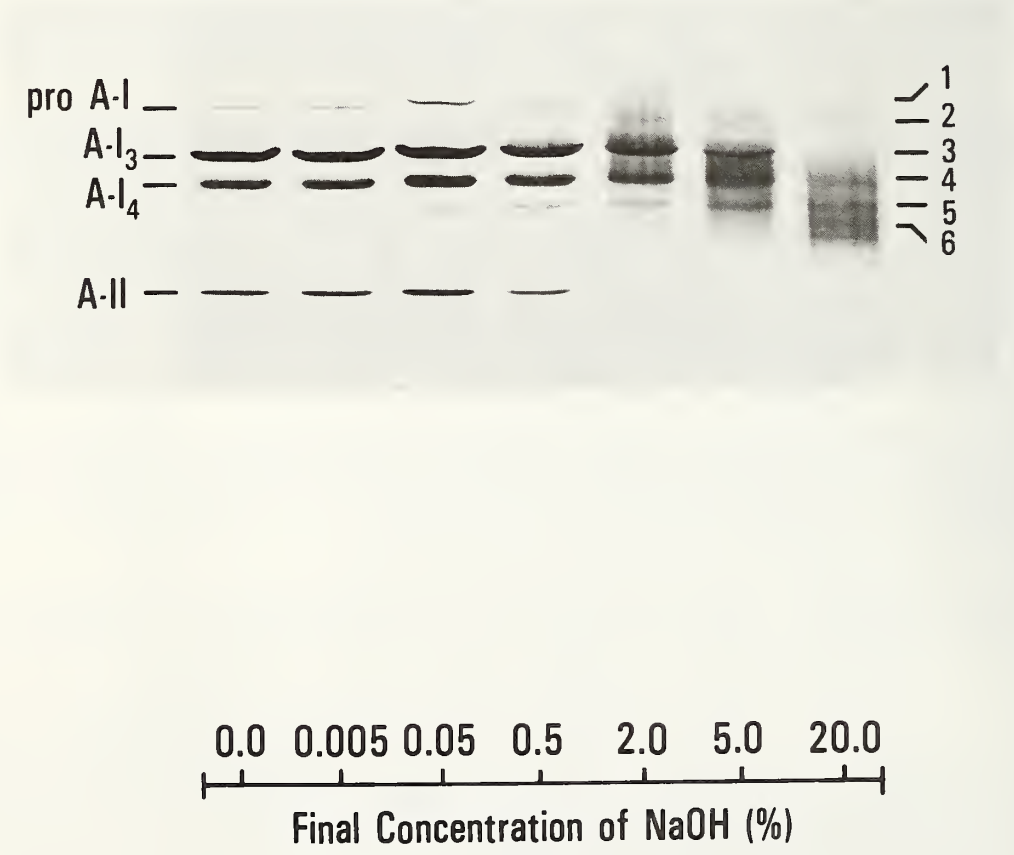

Figure 1. Effect of NaOH treatment on apo A-I isoforms. Isolated HDL at a concentration of 5 mg/ml was exposed to increasing amounts of 1 N NaOH. The pH of the reaction mixtures containing 0.0, 0.005, 0.05, 0.5, 2.0, 5.0 and 20.0% NaOH was 7.1, 7.3, 10.1, 11.64, 12.1, 12.6 and 13.2, respectively. Following neutralization to pH 7.2 with 1 N HCl, 50 ug of HDL was applied to a 6% polyacrylamide gel containing 8 M urea and 2% ampholines, pH 4 to 6. The separated proteins were fixed, stained with Coomassie Blue R-250, destained and photographed. Isoform nomenclature used is that of Ghiselli et al. (4).

amounts of the minor apo A-I isoforms, including pro apo A-I ($A\text{-}I_1$) and $A\text{-}I_2$. HDL treated with increasing concentrations of NaOH were altered in two ways. First, the distinctly staining protein bands became much more diffuse to the extent that HDL treated with 20% NaOH no longer contained discretely stained isoforms. Second, increased amounts of stained material were observed in isoforms $A\text{-}I_4$, $A\text{-}I_5$ and $A\text{-}I_6$ (i.e., all of the protein-staining material became more acidic), suggesting the acquisition of a net negative charge as would occur upon deamidation of asparagine and glutamine residues to aspartic acid and glutamic acid residues, respectively.

HDL treated for 18 hr at a final concentration of 5% NaOH was compared with control HDL with respect to the binding of the three previously characterized apo A-I-specific monoclonal antibodies, AI-4, AI-7 and AI-9 (1). Changes in immunochemical reactivity were monitored by comparing control and NaOH-treated HDL as competitors in solid-phase RIAs. Striking differences in the reactivity of control and NaOH-treated HDL were observed with each antibody (Figure 2). The amount of HDL required to achieve 50%

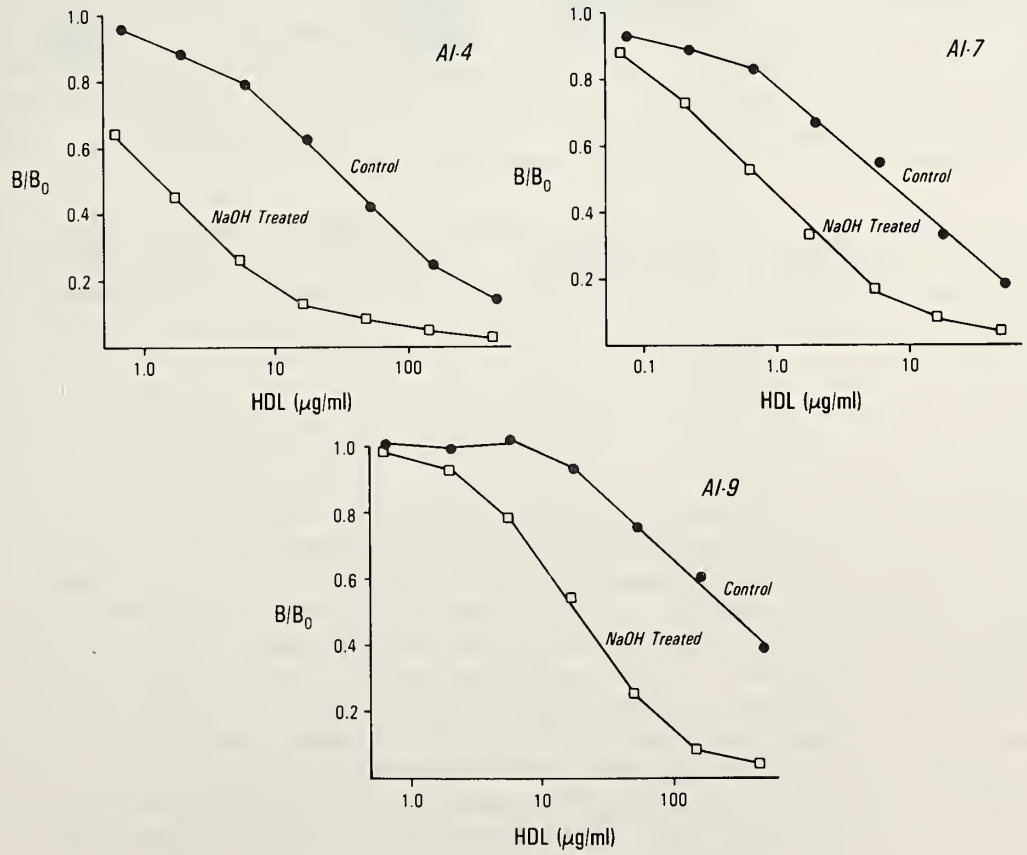

Figure 2. NaOH treatment of HDL increases its immunoreactivity for antibodies AI-4, AI-7 and AI-9. NaOH-treated and control HDL were added as competitors to solid-phase RIAs as described in Methods. The binding characteristics of antibodies AI-4, AI-7 and AI-9 have been reported (1).

inhibition of binding of antibody AI-4 dropped from 35 μg/ml to 1.3 μg/ml, a 27-fold decrease. With NaOH treatment, the amount of HDL required for 50% inhibition of binding of antibody AI-7 dropped from 6.4 μg to 0.75 μg/ml, an 8.5-fold decrease; and for antibody AI-9 it dropped from 260 to 18 μg/ml, a 14.4-fold decrease. Total protein recovery in control and NaOH-treated HDL was 96% and 88%, respectively. Thus, differences in protein recovery could not account for the vast differences observed in immunochemical reactivity. The data suggested that NaOH treatment increased the expression of each of the epitopes on apo A-I recognized by antibodies AI-4, the AI-7 and the AI-9.

In an earlier report, evidence was presented that each of the epitopes bound by these three antibodies were unique epitopes on apo A-I (1). Confirmation that these antibodies bound distinct regions on apo A-I was obtained by identifying that each antibody bound to a separate spectrum of apo A-I CNBr fragments (Figure 3). Antibody AI-4 bound an epitope expressed on CNBr fragments 1 and 1,2; whereas antibody AI-9 bound an epitope on CNBr fragment 4, fragment 3,4 and fragment 2,3,4. Thus, antibody AI-4 was specific for an epitope on CNBr fragment 1 and antibody AI-9 was specific for an epitope on CNBr fragment 4. The binding of antibody AI-7 was less well defined. However, it appeared to bind to a series of CNBr fragments containing either CNBr fragments 2 or 3, because numerous fragments were bound, and this binding was similar, but not identical, to the binding obtained with a polyvalent apo A-I-specific mouse antiserum (Figure 3). The three distinct patterns of immunoreactivity verified that each antibody bound a distinct apo A-I epitope. Thus, there were multiple antigenic determinants on apo A-I that were sensitive to deamidation.

Antibodies AI-4, AI-7 and AI-9 were generated using either isolated and stored apoproteins as immunogens, or HDL that had been isolated on a single occasion from a individual and then stored at 4°C over the course of immunization, a process that could last up to 3 months. This later protocol was used because we wished to screen these antibodies for their ability to detect apo A-I allotypes. However, because each antibody detected an epitope whose expression was increased by alkaline treatment (a condition that leads to deamidation of apo A-I) and because deamidation occurs upon storage of apo A-I <u>in vitro</u>, we chose to generate additional monoclonal antibodies. This time special care was taken to use only freshly isolated native HDL as an immunogen. Three separate fusions were performed with the spleens of mice that been immunized with fresh native HDL or fresh glutaraldehyde cross-linked HDL. Each of the fusions was screened first for hybridomas producing antibodies that bound native HDL coated onto the plastic immunoassay plates. All positive culture supernatants were then screened in fluid-phase immunoprecipitation assays for their capacity to bind all HDL. Four antibodies, selected for their ability to indirectly immunoprecipitate maximal amounts of radioiodinated HDL, were found to be specific for apo A-I (Figure 4). Western blot analysis demonstrated that each of the new antibodies, designated AI-10, AI-11, AI-13 and AI-14, bound apo A-I following the transfer of electrophoretically separated HDL apoproteins to nitrocellulose (data not shown).

When the apo A-I CNBr fragment specificity of these new antibodies was assessed, antibodies AI-11, AI-13 and AI-14 had a CNBr fragment specificity that was identical to that of antibody AI-4. That is, they each bound an

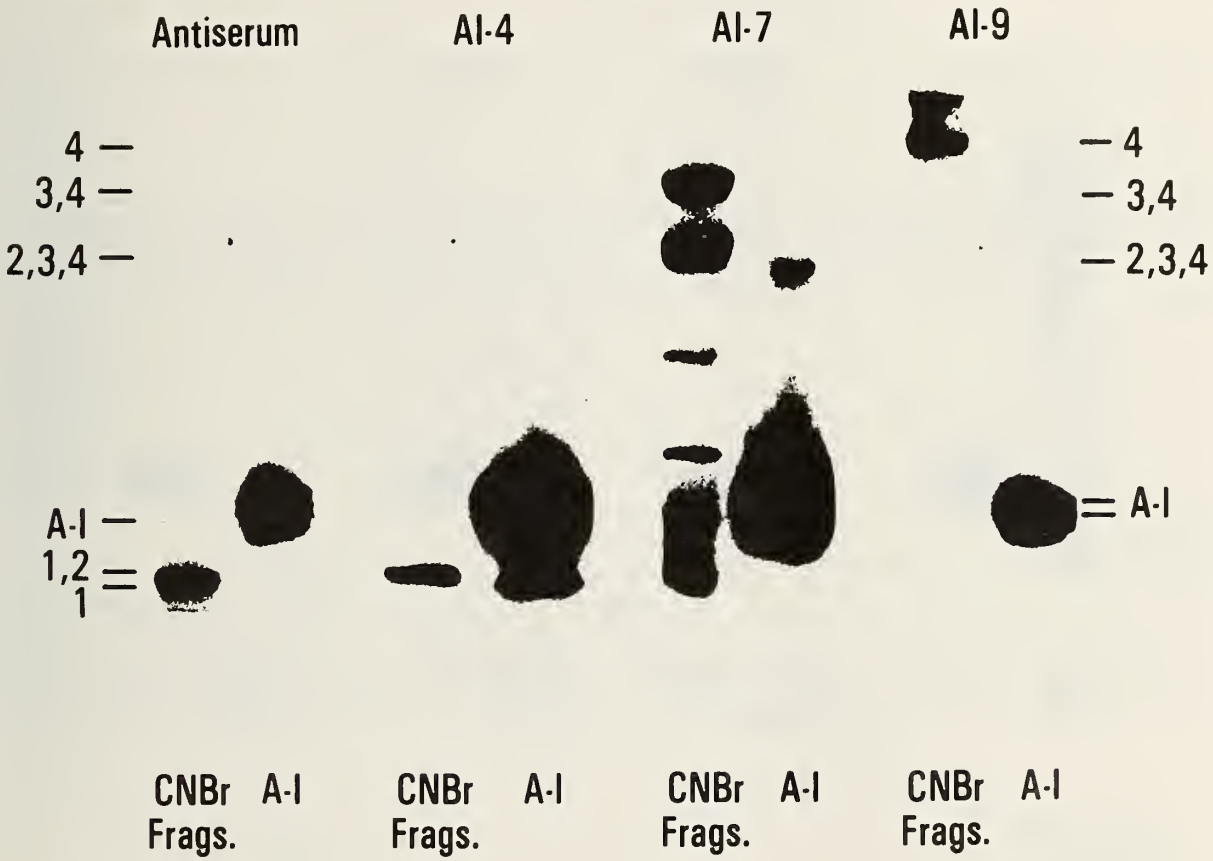

Figure 3. Antibodies AI-4, AI-7 and AI-9 bind unique epitopes on apo A-I. Isolated apo A-I was subjected to CNBr fragmentation as described in Methods, and 25 μg applied to a 6% polyacrylamide isoelectric focusing gel containing 8 M urea. Control, nontreated isolated apo A-I (10 μg) was electrophoresed as well. Following separation the peptides were blotted onto nitrocellulose and incubated with 1:1,000, 1:2,000, 1:4,000 and 1:1,000 dilutions of a mouse anti-apo A-I polyvalent antiserum, or AI-4, AI-7 and AI-9 ascites fluids, respectively. Shown in the figure are photographs of 24 hr autoradiographs obtained after incubation with ^{125}I-goat anti-mouse Ig. The antiserum was included to visualize all apo A-I CNBr fragments. CNBr fragment nomenclature used is that of Rall et al. (9).

epitope on CNBr fragment 1 (Figure 4). Antibody AI-10 bound multiple CNBr fragments, and gave a binding pattern that was essentially identical to that observed earlier with antibody AI-7. Autoradiographs of the Western blots with each of these antibodies can be seen in Figure 4.

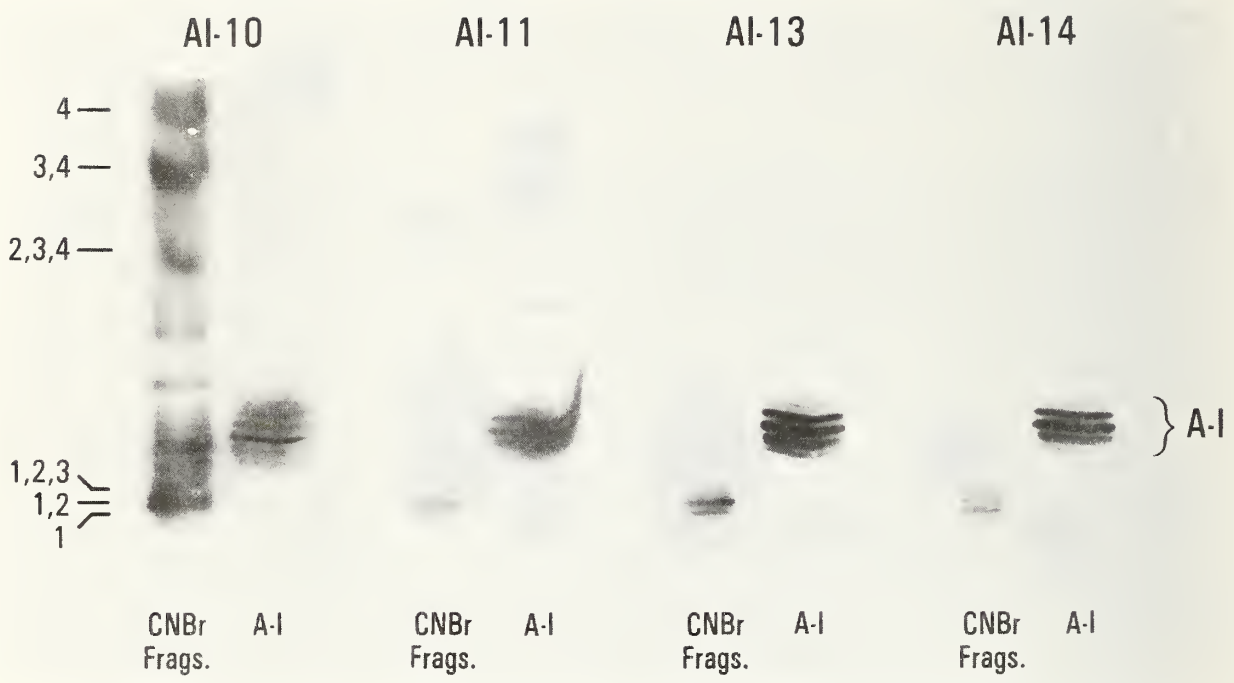

Figure 4. Binding of antibodies AI-10, AI-11, AI-13 and AI-14 to apo A-I and apo A-I CNBr fragments. Apo A-I (10 μg) and CNBr fragmented apo A-I (25 μg) were electrophoresed on a 6% polyacrylamide isoelectric focusing gel containing 8 M urea and 2% ampholines (pH 4 to 6), and then transferred to nitrocellulose for reaction with antibody. Antibodies AI-10, AI-11, AI-13 and AI-14 ascites fluids were used at dilutions of 1:3,000, 1:2,500, 1:4,000 and 1:1,000, respectively. Shown in figure are photographs of 24 hr autoradiographs obtained after incubation of the blots with ^{125}I-goat anti-mouse Ig.

In fluid-phase radioimmunoassays performed with fresh ^{125}I-HDL, the new antibodies, AI-10, AI-11, AI-13 and AI-14, bound between 92% and 100% of radioiodinated HDL (Table 1). These results were in sharp contrast to results obtained earlier with antibodies AI-4, AI-7 and AI-9, where maximum binding of ^{125}I-HDL was consistently less than 60% of HDL (1). It should be emphasized that the binding of HDL reached 100% with only one antibody. Furthermore, it was not known if the difference of 8% to 7% observed with the other antibodies represented technical inconsistencies, or true heterogeneity of these particular apo A-I determinants in HDL.

Table 1. Maximum binding of ^{125}I-HDL[a]

Antibody	Hybridoma	Immunogen	^{125}I-HDL Bound (%)
AI-10	H91H4	glutaraldehyde HDL[b]	92.6
AI-11	H103D8	Native HDL	100.0
AI-13	H105F4	Native HDL	93.1
AI-14	H114D12	Native HDL	91.4

[a]Maximum binding was determined in fluid-phase immunoprecipitation RIAs containing a final concentration of 66.7 ng/ml of ^{125}I-HDL.
[b]The glutaraldehyde cross-linked HDL was prepared with fresh HDL.

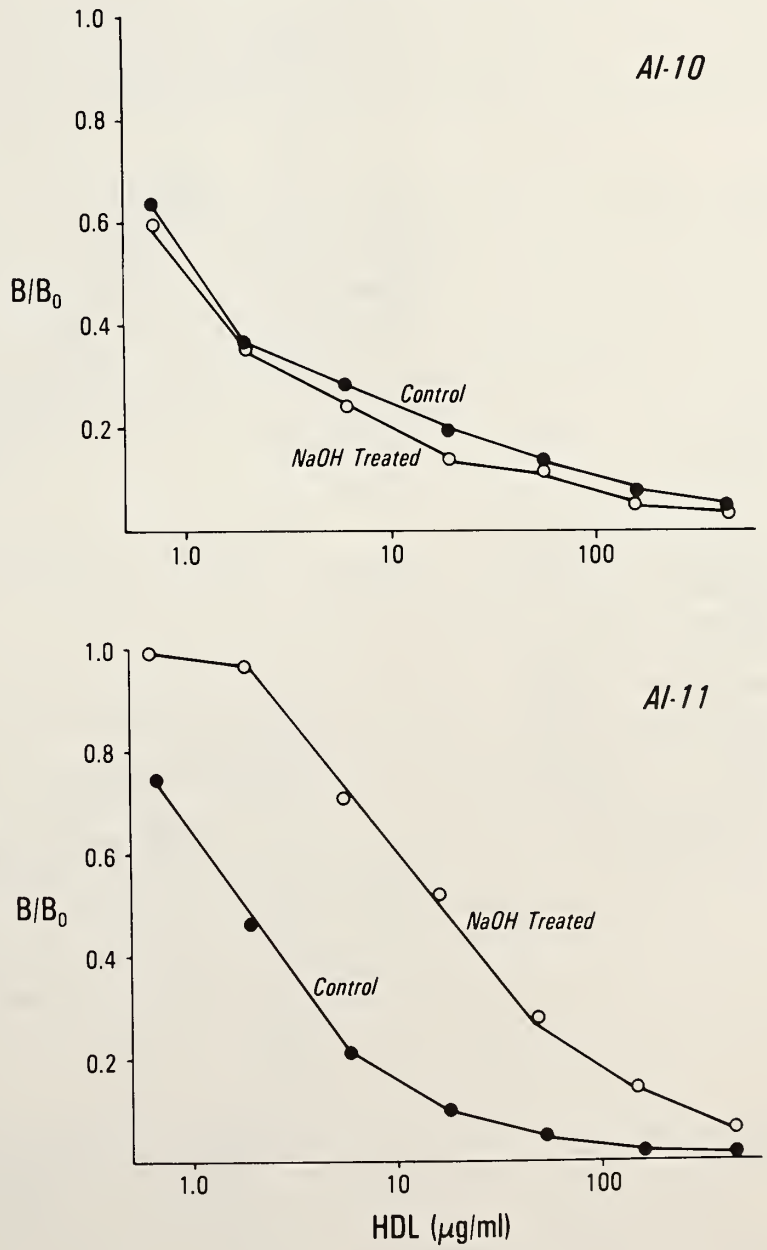

Figure 5. NaOH treatment of HDL has no effect on the reactivity of antibody AI-10 and decreases the immunoreactivity of antibody AI-11. The isolated fresh HDL was treated for 18 hr at room temperature at a final concentration of 5% NaOH (pH of 12.6). Following neutralization to pH 7.2 with 1 N HCl, control and NaOH-treated HDL were added as competitors to solid-phase RIAs performed with fresh HDL-coated immunoassay plates as described in Methods.

The effect of NaOH treatment of HDL on the binding of the new antibodies was examined as described earlier using solid-phase competitive RIAs. Representative results are shown in Figures 5 and 6. Essentially all three possible effects were observed. The capacity of NaOH-treated HDL to bind antibodies AI-10 (Figure 5) and AI-13 (Figure 6) did not differ appreciably from untreated control HDL, presenting good evidence that the epitopes recognized by these antibodies on apo A-I were not altered by alkaline treatment of HDL. In contrast the binding of antibodies AI-11 (Figure 5) and AI-14 (Figure 6) were altered by NaOH treatment of the HDL. Unlike the binding of the earlier antibodies AI-4, AI-7 and AI-9, NaOH treatment decreased the capacity of HDL to be a competitor for binding to antibody

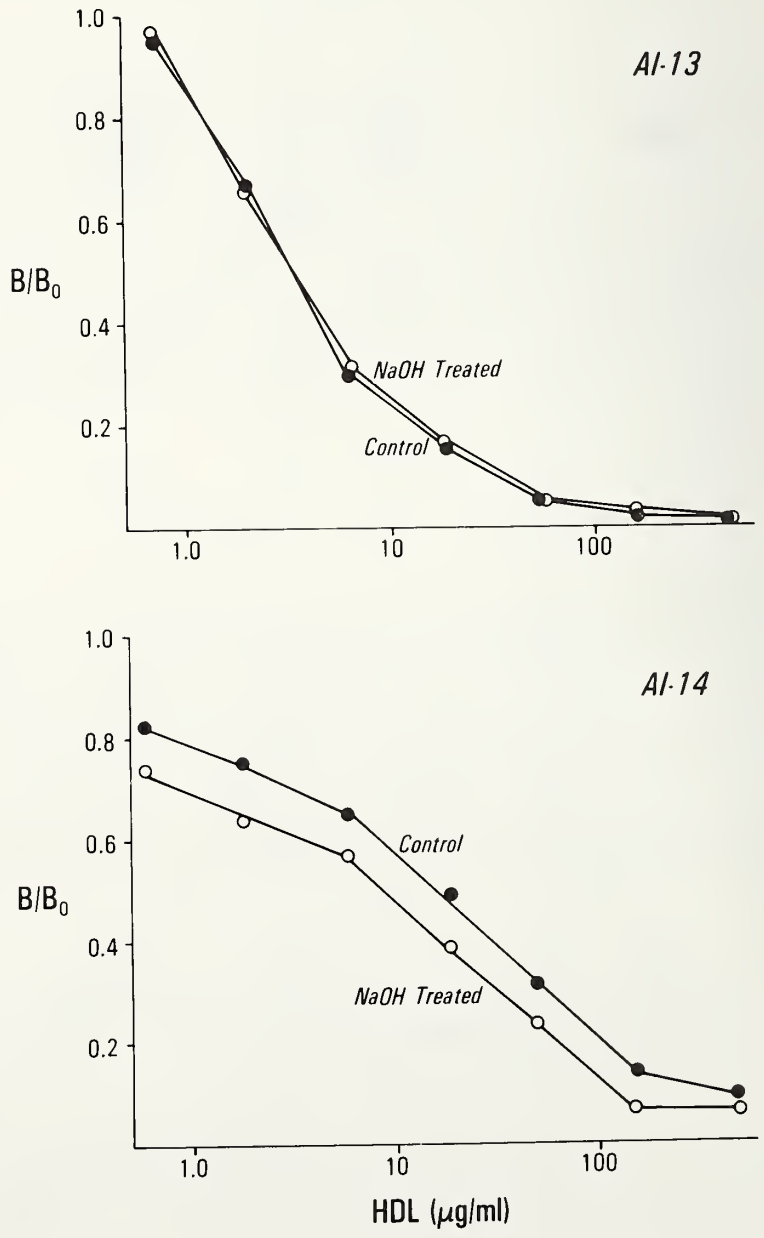

Figure 6. NaOH treatment of HDL has no effect on the reactivity of antibody AI-13 and increases the immunoreactivity of antibody AI-14. Control and NaOH-treated HDL were prepared and assayed as described in the legend of Figure 5.

AI-11. In fact, NaOH treatment caused 8.8-fold decrease in the amount of HDL required to inhibit the binding of antibody AI-11 by 50%. This suggested that the epitope identified by antibody AI-11, in contrast to the "deamidated" epitopes identified by the earlier antibodies, represented a "fresh" or "amidated" epitope on apo AI. Antibody AI-14 appeared to bind a "deamidated" epitope on HDL, because its immunoreactivity for HDL, like that of antibodies AI-4, AI-7 and AI-9, was increased by NaOH treatment (Figure 6).

A summary of the effect of NaOH treatment of HDL on the immunoreactivity of each apo A-I antibody is given in Table 2. Here the antibodies are grouped according to their apo A-I CNBr fragment binding patterns. The four antibodies specific for epitopes on CNBr fragment 1, represented all possible observed effects of HDL alkaline treatment including increased reactivity (↑), decreased reactivity (↓) and no effect (-). The two antibodies that bound all CNBr fragments containing fragments 2 or 3, antibodies AI-7 and AI-10, had differing reactivities for NaOH-treated HDL as well (Table 2). Whereas the binding of AI-7 was increased, the binding of AI-10 was essentially unchanged.

Because all of the new antibodies did not bind 100% of radioiodinated HDL, it was still necessary to verify that the 6% to 9% of the radioiodinated ligand which was not bound by a single antibody contained apo A-I. This was verified by collecting the supernatants from the immunoprecipitation reaction mixtures and electrophoresing them on SDS polyacrylamide gels. Autoradiography of these electrophoresed supernatants from the fluid-phase precipitation assays with antibodies AI-10, AI-13 and AI-14 confirmed that the unbound material contained both apoproteins A-I and A-II (data not shown).

Table 2. Summary of the effects of NaOH treatment of HDL on monoclonal antibody binding

Antibodies that bind epitopes on apo A-I:		
CNBr 1	CNBr 2, 3	CNBr 4
AI-4 (↑)[a]	AI-7 (↑)	AI-9 (↑)
AI-11 (↓)[b]	AI-10 (-)	
AI-13 (-)[c]		
AI-14 (↑)		

[a](↑)Immunoreactivity for HDL was increased by alkaline treatment of HDL.
[b](↓)Immunoreactivity for HDL was decreased by alkaline treatment of HDL.
[c](-)Immunoreactivity for HDL was not changed by alkaline treatment of HDL.

To determine if combinations of two antibodies could bind 100% of HDL, mixing experiments were performed. Because these studies were performed with fresh HDL, the binding of antibodies AI-4(↑), AI-7(↑), AI-9(↑) and AI-14(↑) were not optimal and as expected none of the possible combinations resulted in 100% binding of fresh HDL. The only other remaining possible combination of two antibodies AI-13(-) and AI-10(-) did not give 100% binding of ^{125}I-HDL. Therefore, with the exception of antibody AI-11, none of the available antibodies or combinations of antibodies could recognize and react with all HDL under conditions of antibody excess.

Discussion

A completely satisfactory explanation for the inability of antibodies AI-4, AI-7 and AI-9 to identify and bind all radioiodinated HDL in immunoprecipitation RIAs could not be found (1). This suggested that both HDL and isolated apo A-I were inherently immunochemically heterogeneous. The studies reported here represent continued efforts to understand the basis for this apparent heterogeneity of apo A-I in HDL. Deamidation of apo A-I has been reported, and Milthrop et al. (11) had reported that alkaline treatment of HDL could alter the binding of some of their apo A-I-specific monoclonal antibodies. We therefore tested the effects of alkaline treatment on our antibodies. Alkaline treatment of HDL, accomplished by overnight incubation of HDL in the presence of 5% NaOH, increased the capacity of HDL to bind each of these older antibodies. Incubation of proteins at alkaline pH and at elevated temperatures results in the deamidation of asparagine and glutamine residues. Deamidation of asparagine to aspartic acid or glutamine to glutamic acid converts an uncharged amino acid to an acidic or negatively charged amino acid. Thus, deamidation is characterized by a net increase in the isoelectric point of a given protein (4). We demonstrated that NaOH treatment of HDL resulted in the increase in apo A-I isoforms having a more negative charge (Figure 1). Extreme alkaline conditions resulted in a loss of appearance of specific apo A-I isotypes, as well as a net increase in charge. Although not directly demonstrated in the isoelectric focusing gels, alkaline treatment of HDL resulted in protein fragmentation as well because it could be demonstrated by protein staining of SDS polyacrylamide gels electrophoresis that some of the intact 28,000 molecular weight apo A-I was lost (data not shown).

Because striking differences were found in the immunochemical reactivity of antibodies AI-4, AI-7 and AI-9 to alkaline-treated HDL, we attempted to generate antibodies that were directed towards epitopes which were not altered by alkaline treatment. Among the four new antibodies obtained from three separate fusions of the spleens of mice immunized with fresh HDL, two antibodies were found whose reactivity appeared to be unchanged by NaOH treatment of HDL. Interestingly, a third antibody (AI-11) was found whose immunochemical reactivity decreased upon alkaline treatment of the HDL. This antibody bound CNBr fragment 1 similar to the binding of AI-4, and the possibility exists that AI-4 and AI-11 identify identical regions on apo A-I, but the epitopes differ in that one is directed towards the deamidated form, whereas the other is specific for the amidated form. Mixing experiments involving antibodies AI-4 and AI-11 were not additive, suggesting that this may be the case. Although the binding pattern of antibodies AI-7 and AI-10 were similar, the observation that the binding of antibody AI-7 was drastically affected by NaOH treatment of the HDL and the binding of

antibody AI-10 was not suggests that the epitopes identified by these two antibodies are distinct. The results of the mixing experiments support this.

The fact that antibodies with preferential reactivity for alkaline-treated HDL were generated when stored or "aged" immunogens were used, and antibodies with preferential reactivity for fresh or "amidated" HDL were generated when fresh immunogens were used suggests that deamidation of apo A-I can occur upon storage of HDL or apo A-I. And, while there is no direct evidence that the two phenomenon are exactly equivalent, there is additional evidence that deamidation can occur upon storage of isolated HDL as well as upon storage of HDL in plasma. This has been pointed out by the work of Milthorp, et al. (11), who generated a series of apo A-I-specific monoclonal antibodies that had preferential reactivity for HDL in stored serum. The reactivity of a particular apo A-I antibody termed "3D4" was increased over 50-fold by storage of serum at 4°C for 28 days. This same antibody also had increased reactivity for NaOH-treated HDL, suggesting that at least some of the changes which occur upon storage are mimicked by alkaline treatment. However, they also conclude that alkaline treatment of HDL is only a partial model for antigenic modification of apo A-I that can occur upon storage (11). It is apparent that further chemical changes which occur upon storage must be explored including proteolytic and oxidative changes. The results reported here as well as those reported by Milthorp et al. (11) and in preliminary form by Krul et al. (12) each indicate that the effects of storage of apo A-I, HDL or plasmas must be documented if immunochemical approaches are used to measure total plasma levels of apo A-I. It is readily apparent that only some apo A-I antibodies can be used for this purpose.

The data of Ghiselli et al. (4) demonstrated that deamidation occurred *in vivo* at slow rates. If so, one would expect that at any given point in time a certain percentage of apo A-I in plasma would be deamidated. Therefore, it is interesting to speculate that some of these antibodies could be useful in quantitating the various apo A-I isoforms as they exist *in vivo*. This could be particularly useful if, as reported (4), there are differences in the plasma clearance rates of the different apo A-I isoforms.

Finally, it is interesting to speculate on the location of the particular determinants of these antibodies on apo A-I using the information that some of these antibodies are effected by alkaline treatment of HDL. Meinwald et al. (5) have demonstrated using synthetic peptides that in mildly alkaline solutions the asparagine-glycine sequence is readily deamidated. Next in order of reactivity are other asparagines followed by glutamines. This information and the known CNBr reactivity of each of the monoclonal antibodies can be used to predict which regions of the primary protein sequence of apo A-I have a high probability of representing the epitopes bound by each antibody. With this data synthetic peptide fragments can be prepared and used to identify the antigenic determinants recognized by this panel of apo A-I-specific monoclonal antibodies.

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FUNCTION OF HDL SUBSPECIES IN CHOLESTEROL TRANSPORT FROM CELLS

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Introduction

It is generally believed that HDL functions to mediate cholesterol transport from extrahepatic cells to the liver, a process termed "reverse cholesterol transport" (1). As illustrated in Figure 1 (see Refs. 2 and 3 for review), some of the major subspecies of HDL particles described in this workshop may represent discrete intermediates along this lipoprotein pathway. Sterol-deficient HDL particles can act as acceptors for free cholesterol derived either from cell membranes (step 1, Fig. 1) or the surface of other circulating lipoproteins (step 2, Fig. 1). The free cholesterol sequestered by these particles can then be converted to cholesteryl esters by the enzyme lecithin cholesterol acyltransferase, leading to an increase in core volume. At one or several steps along this pathway, free and esterified cholesterol may be transported directly to the liver for removal from the blood by processes still largely unknown. Alternatively, cholesteryl ester may be transferred to apo-B-containing lipoproteins in exchange for triglyceride as catalyzed by cholesteryl ester transfer protein. This transfer process leads to regeneration of small, precursor particles, since the triglyceride incorporated into the core of HDL can be readily hydrolyzed by lipoprotein and hepatic lipases.

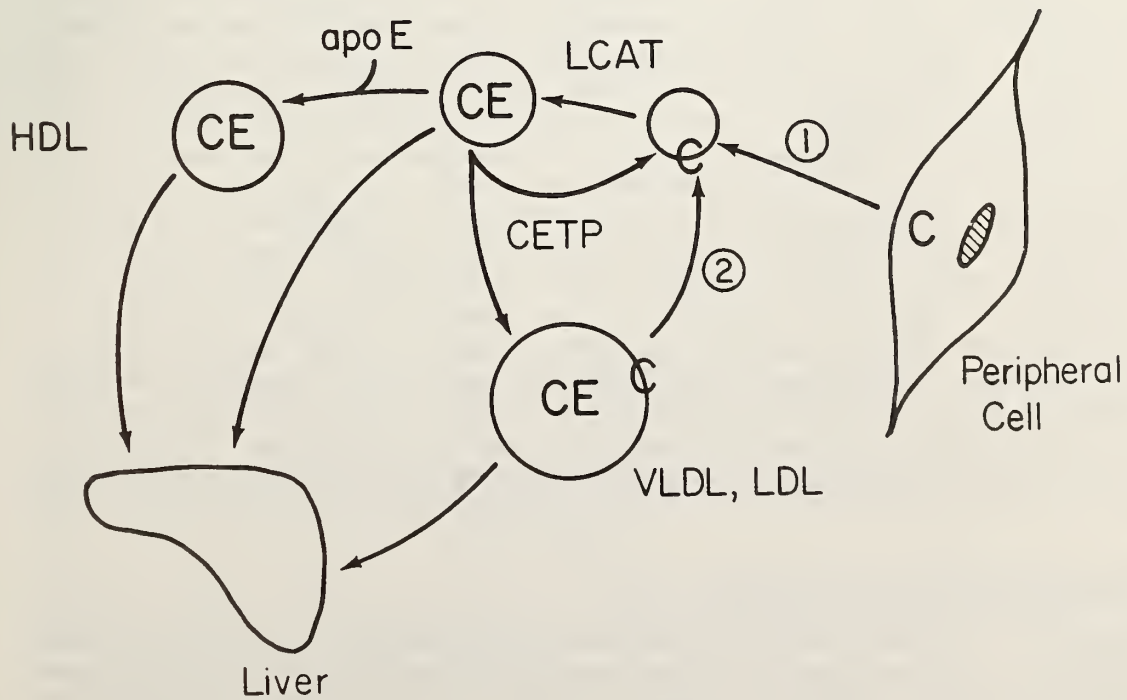

Figure 1. A schematic representation of reverse cholesterol transport.

Although it is now apparent that multiple subspecies of HDL particles exist in plasma, very little is known about the metabolic role of these subspecies. The current paper will focus on the potential role of HDL subspecies in the removal of cholesterol from extrahepatic cells, the first step in the pathway of reverse cholesterol transport. The importance of this step as a potential modulator of flux of cholesterol through the entire pathway was made apparent by recent studies suggesting that removal of cholesterol from cells by HDL is mediated by binding of HDL to a specific cell-surface receptor.

The HDL Receptor Hypothesis

Studies from many different laboratories have demonstrated that a wide variety of cell types possess high-affinity binding sites in their plasma membranes that specifically interact with HDL. Studies from our laboratory suggest that at least one class of these sites represents true physiologic receptors for HDL that act to facilitate transport of cholesterol from cells to HDL particles. This HDL receptor hypothesis is based on results demonstrating that cholesterol enrichment of cells in tissue culture leads to an increase in HDL binding to cells by a process that appears to involve synthesis of cellular protein (4,5). Recent ligand-blotting studies have demonstrated that cellular membranes contain a 110-kilodalton protein that binds HDL_3 (6). As is characteristic of the binding site on intact cells, this binding protein specifically interacts with the major HDL apoproteins, apo A-I and A-II, and its binding activity is increased when cells become loaded with cholesterol. Taken together, these results provide evidence that cellular membranes contain a receptor protein that plays an important role in modulating the rate of flux of cholesterol from cells to HDL particles.

Although receptor binding of HDL may not be necessary for cholesterol transport from most cells under steady-state conditions, it may facilitate HDL-mediated removal of cholesterol from cells when they become overloaded with cholesterol. Tissue culture studies have shown that quiescent cells have higher levels of HDL receptor activity than rapidly growing cells, even when the cell cholesterol content is low (7). Since quiescent cells require little cholesterol for membrane synthesis, they probably maintain high levels of HDL receptors to facilitate continual removal of excess cholesterol from cells. Because confluent aortic endothelial cells are in a growth-arrested state, their membranes contain a high concentration of HDL receptor molecules (5,6). This observation raises the possibility that circulating lipoproteins may interact directly with HDL receptors on the luminal surface of arteries *in vivo*. This interaction could be part of a branch pathway whereby continual flux of cholesterol from sterol-rich lipoproteins to HDL particles occurs via endothelial cell pathways involving one or more lipoprotein receptors. Thus, it is of interest to identify and characterize the plasma HDL particles that directly interact with cell-surface receptors and facilitate removal of cholesterol from cells.

Parameters of Cholesterol Transport

Movement of cholesterol between cells and HDL particles is bidirectional, containing both an efflux and an influx vector. Thus, the rate of net transport of cholesterol in either direction is defined as the difference between efflux of cellular cholesterol to HDL particles and influx of HDL cholesterol into cells. To measure efflux of cholesterol under conditions where HDL

receptor activity is upregulated, cultured cells are loaded with [^{3}H]cholesterol and the rate of appearance of radioactivity in the medium is measured (Fig. 2, left). The ideal way to measure influx of cholesterol from HDL is to radiolabel the free cholesterol pool in HDL particles and monitor movement of radioactivity into cells. However, this procedure is not practical when comparing different populations of HDL particles. A simpler approach is to measure net transport of cholesterol from cells, since cells incubated under identical conditions can be used to screen HDL samples of variable lipid composition. Because the amount of cholesterol transported from sterol-laden cells during the first 4 hours of incubation can represent less than 5% of the total cell cholesterol content, we use a sensitive biochemical marker as an indirect measurement of changes in cell cholesterol content; i.e., the activity of the cholesterol esterifying enzyme, acyl coA cholesterol acyltransferase (ACAT). The activity of this enzyme is a direct function of the amount of excess free cholesterol that accumulates in cells (4,5,8,9). A 5% decrease in excess cellular cholesterol can cause a several-fold reduction in ACAT activity. When comparing different populations of HDL particles, the relative difference in cholesterol influx between these populations can be deduced from measurements of both cholesterol efflux and net cholesterol transport (Fig. 2, right).

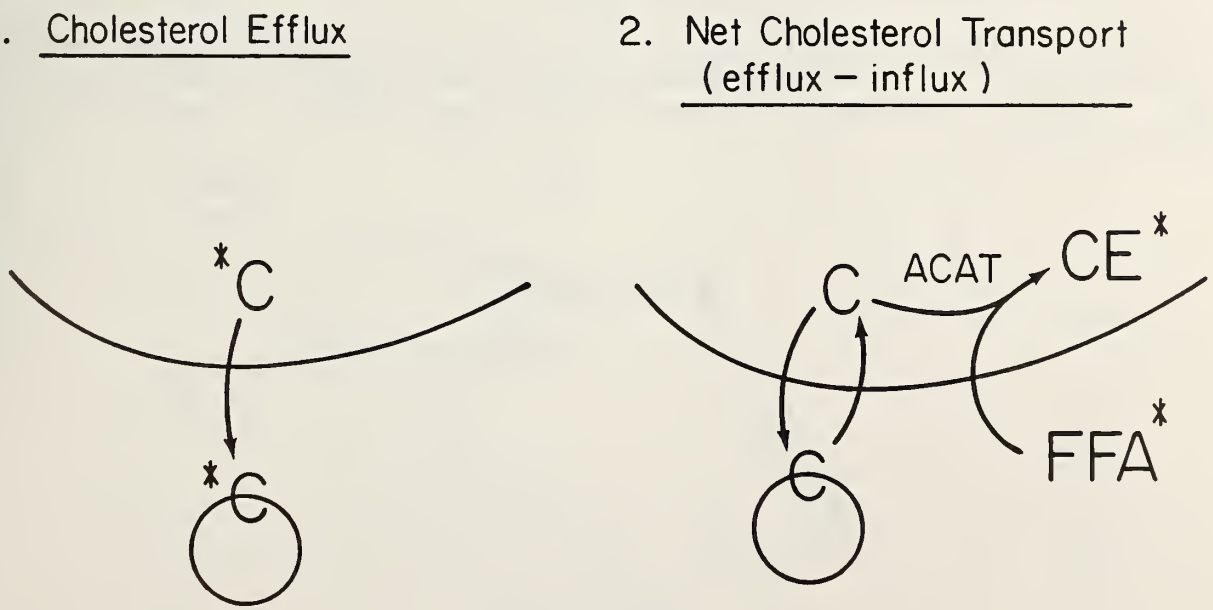

Figure 2. Assays for cholesterol transport from cells. Asterisk indicates radiolabeled molecules.

Reduction in Cellular Binding and Cholesterol Transport from Cells by Nitration of HDL Protein

Modification of HDL apoprotein by treatment of lipoprotein with tetranitromethane (TNM) leads to a dose-dependent decrease in its cellular binding activity (Fig. 3). This occurs at concentrations of TNM (1 to 3 mM TNM at 1 mg/ml HDL_3) where only modest changes in lipid composition and particle morphology are evident (9,10). Over the same range of TNM concentrations that inhibited cellular binding of HDL, the ability of HDL_3 to promote both efflux and net transport of cholesterol from fibroblasts was reduced (Fig. 4). These results provide evidence that both efflux and net transport of cholesterol from cells to HDL_3 is facilitated by binding of HDL to its cell-surface receptor.

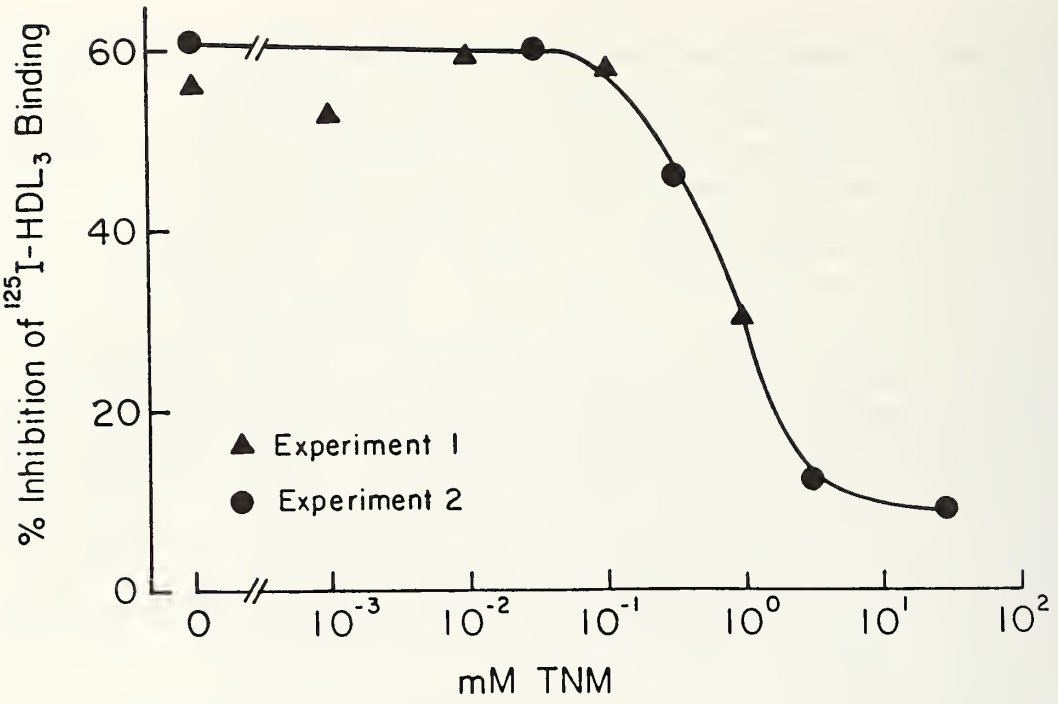

Figure 3. Effect of treatment of HDL_3 (1 mg protein/ml) with increasing concentrations of tetranitromethane (TNM) on its ability to compete for ^{125}I-HDL_3 binding to cholesterol-loaded fibroblasts. (Modified from reference 9 with permission.)

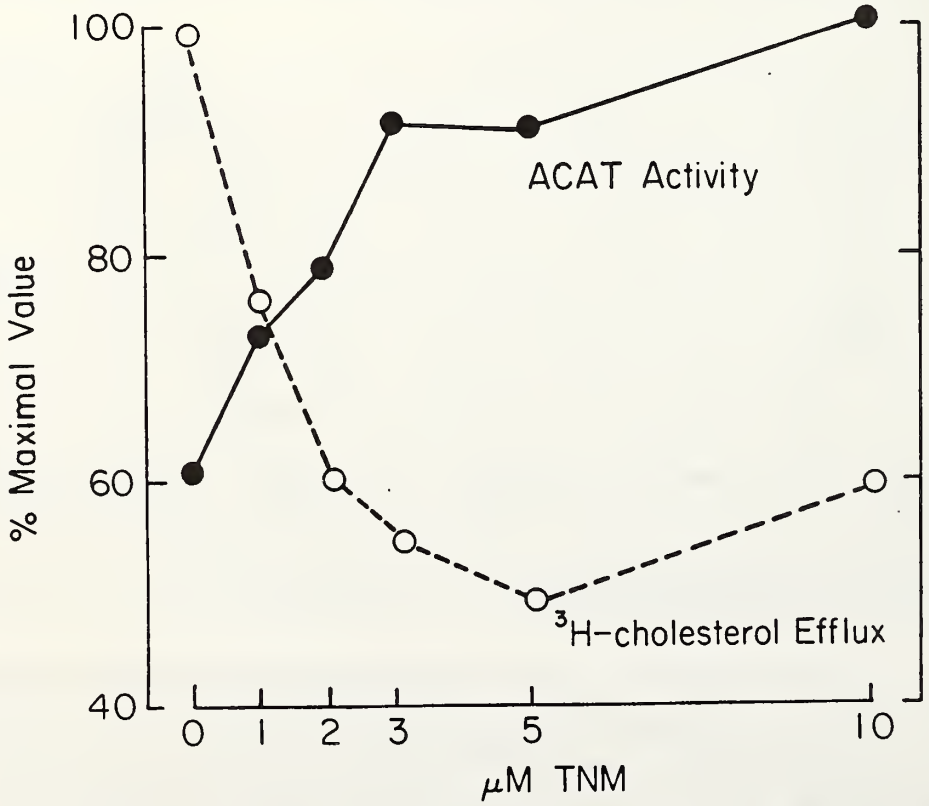

Figure 4. Effect of TNM treatment of HDL_3 on its ability to promote ^{3}H-cholesterol efflux (o) or net cholesterol transport (•) from cholesterol-loaded fibroblasts during 4 h incubation at 37°C. (Modified from reference 9 with permission.)

Cellular Binding and Promotion of Cholesterol Transport from Cells by Apoprotein-phospholipid Vesicles

Egg lecithin liposomes prepared in the presence of albumin are poor competitors for ^{125}I-HDL$_3$ binding, suggesting that they do not interact with HDL receptors. In contrast, liposomes prepared with apo A-I compete avidly for HDL$_3$ binding, implying that the HDL receptor recognizes phospholipid vesicles that contain this apoprotein. Apo A-I vesicles are also much more effective than apoprotein-free vesicles in promoting both efflux and net transport of cholesterol from cells (Fig. 5). These results provide additional support for the hypothesis that binding of particles to the HDL receptor facilitates removal of cholesterol from cells.

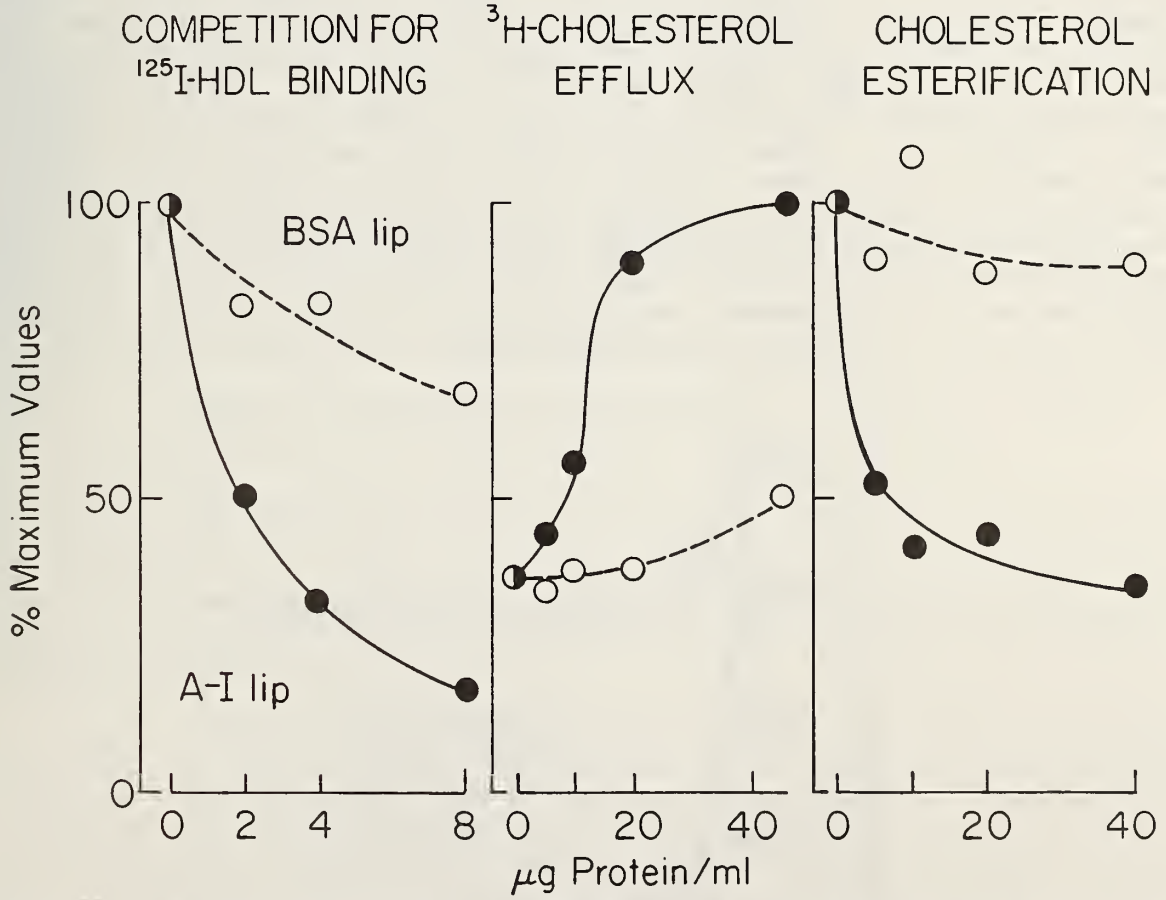

Figure 5. Cellular binding and promotion of efflux and net transport of cholesterol from fibroblasts by proteoliposomes. Egg lecithin proteoliposomes were prepared by cholate dialysis in the presence of either bovine serum albumin (BSA) or apoprotein A-I at a lecithin-to-protein molar ratio of 180:1 (9). Assays were performed as described in Figures 3 and 4.

Cellular Binding and Promotion of Cholesterol Transport from Cells by Different Subfractions of HDL

The studies described in the previous two sections compared cellular interactions of populations of particles that were different in apoprotein composition or structure but were similar in lipid composition. The two major

subclasses of HDL, HDL_2 and HDL_3, contain similar amounts of apoprotein, but the relative lipid composition of the particles are different. The total core volume of HDL_2 is about 3.5-fold larger than that of HDL_3, but the surface area of HDL_2 is only twice that of HDL_3. Therefore, HDL_2 contains about 3.5 times the cholesteryl ester content but twice the phospholipid content of HDL_3. The free cholesterol content of HDL_2 is up to five times that of HDL_3, despite only twice the surface area. As much as one-third of the total HDL_2 cholesterol is in the free form (11). With this high a free cholesterol content, it can be predicted that flux of cholesterol from HDL_2 particles into cells would be a significant process.

A comparison of the competitive binding activity of HDL_2 and HDL_3 revealed that, on a protein basis, both types of particles have the same receptor binding activity (Fig. 6). Treatment of particles with TNM reduced binding activity of both classes of lipoprotein to the same extent. When subjected to cholesterol assays, both HDL_2 and HDL_3 had the same ability to promote cholesterol efflux, but only HDL_3 caused net transport of cholesterol from cells (Fig. 7). These results indicate that receptor binding activity and promotion of cholesterol efflux by HDL are both independent of particle size and lipid composition. However, the ability of an HDL subpopulation to promote net transport of cholesterol from cells is influenced by the lipid composition of the particles. Since there was no difference in cholesterol efflux between HDL subclasses under conditions where net cholesterol transport was different, HDL_2 appeared to promote influx of cholesterol into cells.

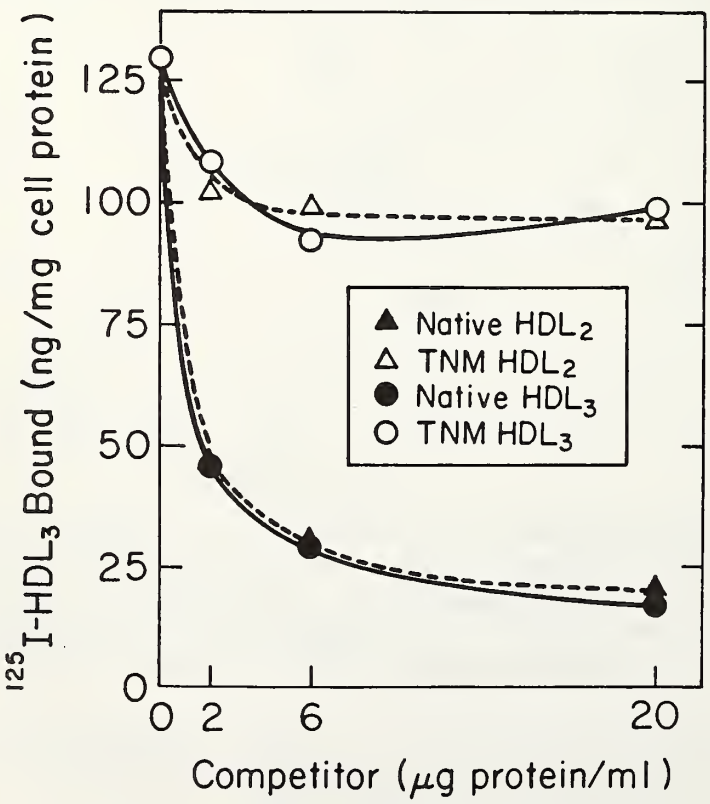

Figure 6. Competition of untreated and TNM-treated HDL_2 and HDL_3 for binding of ^{125}I-HDL_3 to cholesterol-loaded fibroblasts. (From reference 9 with permission.)

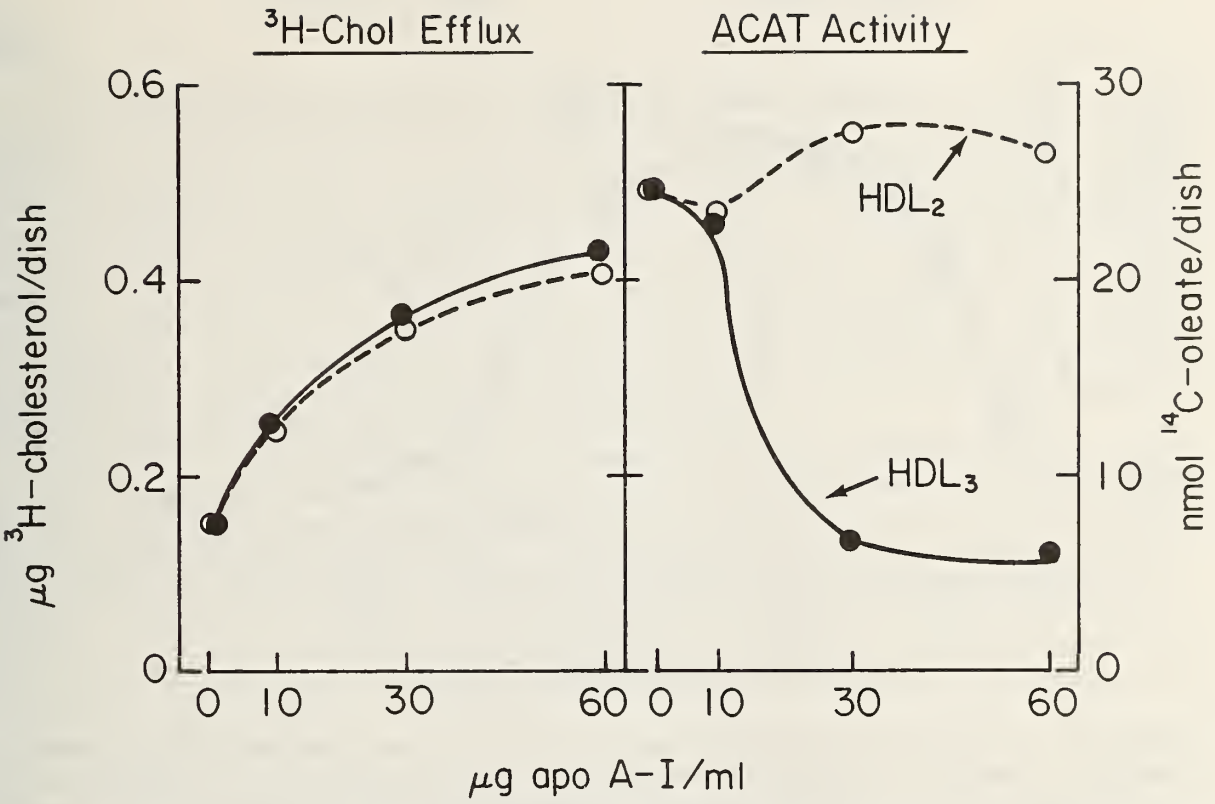

Figure 7. Promotion of cholesterol efflux and net cholesterol transport (ACAT activity) from cholesterol-loaded fibroblasts by HDL_2 and HDL_3.

Delivery of cholesterol into cells by uptake of whole HDL_2 particles or core lipids did not play a significant role in the transport process, since addition of the lysosomal enzyme inhibitor chloroquine had no effect (12). It is likely that, because of its relatively high free cholesterol content, HDL_2 promotes influx of cholesterol at a rate that equals or exceeds efflux of cholesterol from cells to HDL_2.

Recent studies have demonstrated that HDL is populated by at least two major subclasses of particles that can be separated by immunoaffinity techniques: particles containing both apo A-I and apo A-II, and particles containing apo A-I but no apo A-II (13). The particles that contain both apoproteins tend to occupy the hydrated density range of HDL_3, while the particles without apo A-II are distributed within both HDL_2 and HDL_3 density ranges. When tested for their abilities to promote cholesterol efflux, both subclasses showed similar results based on apo A-I concentration (Fig. 8). However, the particles without apo A-II were moderately less effective than those with apo A-II in promoting net cholesterol transport from cells. This would be predicted from studies comparing HDL_2 and HDL_3, since the particles without apo A-II occupy proportionately more of the HDL_2 density range than the other subclass. Again, these results support the hypothesis that the HDL lipid composition is an important determinant of the net transport of cholesterol from cells but has no influence on the unidirectional flux of cholesterol from cells to HDL particles. By inference, it is influx of cholesterol from HDL into cells that varies from one HDL subspecies to another. This observation pertains to subpopulations of HDL particles isolated by both density ultracentrifugation and immunoaffinity techniques.

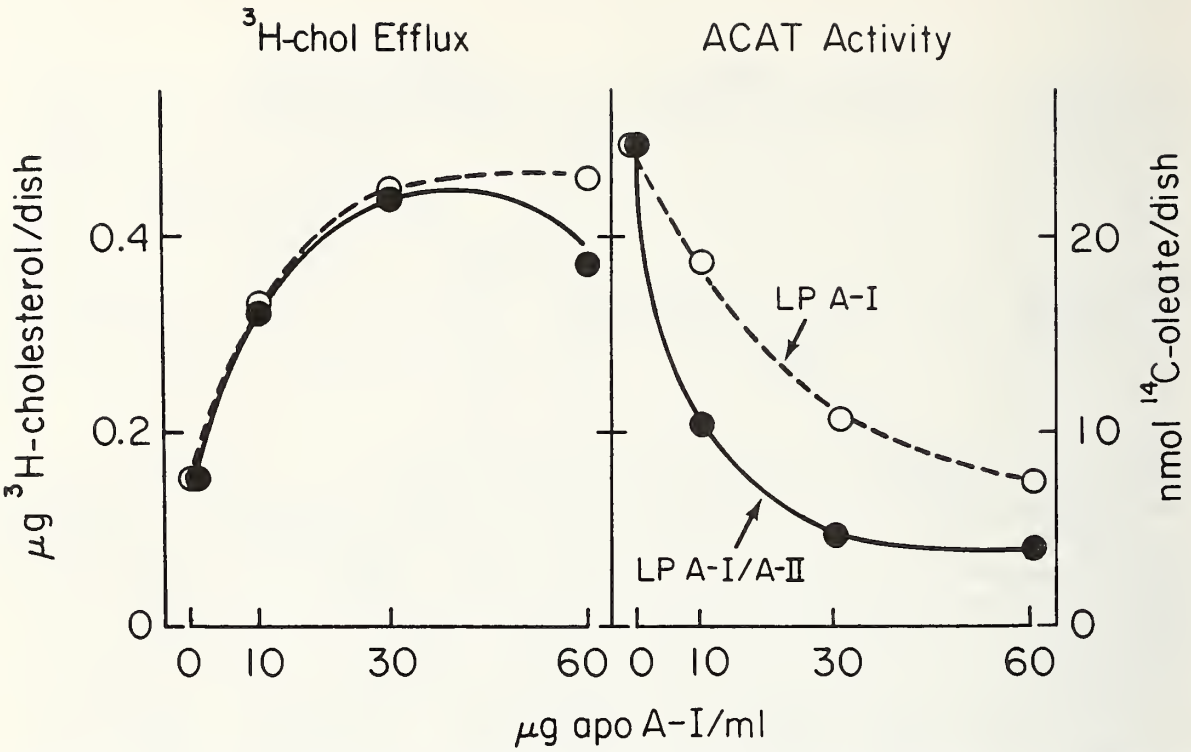

Figure 8. Promotion of cholesterol efflux and net cholesterol transport from fibroblasts by HDL particles isolated from plasma by immunoadsorptive chromatography (13). LP A-I: lipoprotein with apo A-I but without apo A-II. LP A-I/A-II: lipoprotein with both apo A-I and apo A-II.

The Effect of Hypertriglyceridemia on Cellular Binding Activity of HDL Particles and Their Ability to Transport Cholesterol from Cells

An alternative approach to assessing the influence of heterogeneity in lipoprotein lipid composition on the interaction of HDL particles with cells is to compare particles from different individuals who have a wide variation in their HDL lipid composition within the same density range. Hypertriglyceridemia leads to the formation of triglyceride-rich, cholesterol-poor HDL_3 particles that are smaller in size than those from normotriglyceridemic subjects (14,15). We isolated HDL_3 particles from over forty normo- and hypertriglyceridemic subjects and tested them for receptor binding activity and ability to promote cholesterol transport from cells. Statistical analysis of the results demonstrated that neither receptor binding activity nor ability to promote cholesterol efflux from cells was a function of the particle composition of any of the major lipid components in the HDL_3 samples (16). In contrast, the rate of net cholesterol transport from cells showed a significant positive correlation with HDL triglyceride content and a negative correlation with HDL cholesterol content. Thus, this study provided additional evidence that, of the three parameters of cell interaction tested, only net transport of cholesterol was influenced by the heterogeneity in lipid composition that existed between populations of HDL_3 particles. This study also demonstrated a significant positive correlation between cell binding activity of HDL_3 and its ability to promote both efflux and net transport of cholesterol from cells, consistent with the hypothesis that movement of cholesterol between cells and HDL particles is facilitated by binding of HDL to cells.

Summary

There is mounting evidence that cell membranes possess a receptor protein that specifically binds HDL and facilitates transport of cholesterol from cells to HDL particles, the first step in the "reverse cholesterol transport" pathway. Studies using chemically modified HDL_3, apoprotein-phospholipid vesicles, isolated HDL subclasses, and HDL_3 isolated from subjects with different degrees of hypertriglyceridemia, all provided evidence that the lipid composition and size of HDL particles were not determinants of the relative receptor binding activity of these particles. When normalized for either total protein or apo AI content, all subpopulations of HDL appeared to bind to cell receptors to the same extent, suggesting that the recognition site for the HDL receptor lies within the HDL apoproteins.

Similar to what was observed for receptor binding activity, promotion of cholesterol efflux from cells by heterogeneous populations of HDL particles was an independent function of both HDL lipid composition and size. However, the relative ability of HDL particles to promote net transport of cholesterol from cells was dependent on their lipid composition, with cholesterol-rich particles having less transport capacity than cholesterol-poor particles. The ability of a population of particles to promote both efflux and net transport of cholesterol from cells was a function of the receptor binding activity of the HDL particles.

The results of these studies underscore the importance of cellular binding in the HDL-mediated removal of cholesterol from cells. These studies also suggest that subspecies of HDL particles isolated according to density and size are not likely to exhibit differences in their ability to interact with cells. However, these subspecies may contain discrete subpopulations of particles that interact with different affinities toward cellular binding sites, and more sophisticated techniques may be required to identify these particles. For example, Mendel *et al.* (17) reported that an apo A-I-containing lipoprotein with pre-beta electrophoretic mobility has a much lower affinity for binding sites on bovine liver membrane than does typical HDL_3.

Under most conditions *in vivo*, the concentration of HDL particles in the extracellular fluids exceeds that needed to saturate HDL receptors on cells (>20 μg/ml). Thus, HDL receptor-mediated transport of cholesterol from a particular tissue would be expected to be determined by both the number of cell-surface receptors and the binding affinity of the HDL subspecies that have access to those receptors. However, the steady-state level of cholesterol within cells would also be influenced by the lipid composition of the total population of HDL particles in the extracellular environment. This in turn depends on the activities of enzymes, such as lecithin cholesterol acyltransferase and lipoprotein and hepatic lipases, as well as lipid transfer proteins that act to regenerate cholesterol-depleted particles. It is apparent that the degree of interaction of a particular HDL subspecies with multiple cellular and extracellular components will have to be considered when assessing the function of that subspecies in transporting cholesterol from and to different tissues.

Acknowledgements

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DR. PACKARD: The French group in Lisle have been looking at LPA-I and LPA-II levels in several hundred known coronary artery disease patients and they see very clearly that the LPA-I without AII is much lower in coronary disease. It is about half the normal level in coronary disease patients. So, I just wonder whether you'll be able to find a difference as well when the numbers get bigger? Are you convinced there is no difference?

DR. CHEUNG: Let me reinterpret that data that I showed you. The distribution was not different. If you have a subject with a lower HDL, your absolute quantities are going to be different, even with the same distribution. So, I was showing that there was no distribution difference.

DR. PACKARD: I meant in the total plasma level.

DR. CHEUNG: The total plasma level of the two classes we have studied do show a lower A1 in coronary artery disease subjects and they do have a lower HDL; however, the HDL was comparable to the lipid research clinic level for their age and for sex. So they are not really low HDL.

DR. SNIDERMAN: I have a question for Dr. Segrest and Dr. Cheung. Do you concede that the reverse cholesterol transfer pathway is separated from part of the HDL fraction that takes part in TG-cholesterol exchanges when you have changes in HDL inversely related to plasma transfer?

DR. SEGREST: Are you asking if we think that is seen here?

DR. SNIDERMAN: Yes.

DR. SEGREST: I have a particular bias about reverse cholesterol transport and HDL. I think that HDL is reflecting changes in other types of lipoproteins, probably in the post-prandial state. I think HDL reverse cholesterol transport occurs and it probably has some importance. I'm not so sure it is highly important in the prevention of atherosclerosis. I think HDL may be doing something else.

DR. BARTER: Dr. Nichols, you showed the incubation with the various discoidal particles in 24 hours resulted in a fairly homogeneous population of a given diameter. Do you ever see intermediate particles on the way or do you finish up with a given end point from a particular starting point?

DR. NICHOLS: When you look at the transformation of the two A1 particles, those have that quantum step transformation. When you look at the three A1 particles or the four, those have the gradual shift and so in the interim time you see particles appearing on the gradient gel and in great part those are those deformable particles you saw before. So, all of those are conserving their A1 level or number, but in the same breath are changing.

Dr. MELCHIOR: Dr. Cheung showed that the traditional centrifugation methods create artifacts. Dr. Segrest, did your single vertical spin method spin off lipoproteins?

DR. SEGREST: It depends on how long you spin. With the really short 45-minute spin, we've not seen a lot of free A1. With the longer HDL spins of five hours or so, we

do see something. Dr. Cheung and I have just collaborated, we sent her something that had been previously spun by one of our vertical spins, about four and a half hours, and it appears that in the A1 without A2 some free A1 probably is produced. So it depends on how long you centrifuge. I would say, yes, if it is spun a long time.

DR. SCANU: Dr. Cheung, you mentioned that LCAT was assessed with ApoA1-containing particles. If you fractionate by density gradient your A1-containing particles where does the LCAT go? Have you followed the LCAT by immunoblot, for instance? Which particle does it prefer? Is the preference of LCAT for single size particles?

DR. CHEUNG: If you look at the gel filtration column, the LCAT activity is actually found in a very broad sized range.

DR. SCANU: My question is in your gradient gels which now you separate further, if you attempt to find out where by immunoblot, for instance, the LCAT is associated and the same for Dr. Nichols. You are adding LCAT to particles starting with two A1s, three A1s, and four and yet you get these transformations. You imply some kind of association between LCAT. Is there any difference in the affinity of this LCAT for these particles?

DR. NICHOLS: You mean insofar as the starting discoidal particle? We haven't measured that. I think what's implied by the data that I've seen from Dr. Albers and Dr. Cheung is that LCAT is associated with the larger HDL particles.

DR. CHEUNG: When we did immunoblot, we separated with a full 30 percent gradient gel and then used anti-LCAT; the LCAT is mostly in the larger sized particles.

DR. SCANU: By density gradient ultracentrifugation?

DR. CHEUNG: I have never done ultracentrifugation on this.

DR. SCANU: You made a comment about a loss of 30 percent. It depends on which condition. By our method, we see barely three or four percent. Should we say that it depends on which kind of method you use?

DR. ALBERS: It is not only the method, but it is also the type or nature of the particle. Dr. Cheung's data, suggests that the larger A1 without A2 species is particularly labile. We'll have to look at the A1 plus A2 particle. The A1 seems to remain with that particle. So that's a very important distinction.

DR. SCANU: It's unusual, not to see (a) positive in so many people. We have just done studies with our cardiologists and they feel that the midwest is different than the south, but we find at least in peripheral vascular disease patients 70 percent are (a) positive.

DR. SEGREST: These are coronary artery disease patients. I very much suspect that that may be a major factor in peripheral vascular disease.

DR. SCANU: My final comment is that we are very aware that we have to study the HDL several times because the acute phase reaction depends upon when you cut it.

DR. SEGREST: The one group that I showed had not had MIs in eight years. So, they just came in with chest pain, angina or whatever. So that may have been the reason.

DR. HAVEL: Dr. Segrest, when you were showing the slide with distribution of D an LCAT, D looked like it was distributed mainly in the large particles, but LCAT looked like it was distributed mainly in the small particles.

DR SEGREST: Looking at their data, this was done with centrifugation. I'm not sure I totally agree with the LCAT position, it's hard to argue with what they've shown. We found LCAT in the three, four, and five region. It didn't look like it was at the bottom of the tube. It was not spun to the bottom which is why I think--maybe it is just redistributed.

DR. HAVEL: Well, whatever particles LCAT associates with, it is one of the particles that is very labile in centrifugation. That might account for these observations. This question of centrifugal association is very serious for some particles--I think Dr. Albers is saying that--and not so serious for others and nobody's mentioned E here. That's one where that's true also.

DR. ALBERS: We can get back to this topic tomorrow. It is very important we get back to the implications of the different methodologies and where we go from here. That is part of our task.

SPEAKER: Dr. Cheung, a couple of years ago looking at hypercholesterolemia in primates, looking at ApoA2 distribution, in centrifuge, density gradient, I'm a little bit surprised to find significant amounts of A2 in the IDL fraction of the non-human primate and in fact it was a density of 1.016, so true IDL. And it did not correspond with the big cholesterol and ApoB peak. You indicated that you occasionally saw it in VLDL, sometimes your A2 immunoaffinity column picked up some VLDL. Have you seen any larger cholesteryl ester particles that come off the column that are not HDL other than the VLDL? I'm asking if you ever see IDL?

DR. CHEUNG: I'm not in the position to even tell you what density the particles are since I never put them through a centrifuge, but on the gradient gel we do see particles larger than the biggest size standard that we have which is 17 nanometers. So, I would assume above that would be anything between HDL1 or IDL, LDL or VLDL.

DR. SEGREST: When we do this same vertical spin on whole plasma and do radioimmunoassays on fractions there is no A2 in the fraction that floats to the top which is LDL, IDL, VLDL. We've never seen any up there. It may have spun off.

DR. GINSBERG: Dr. Curtiss, a question, one that is peripheral to 99 percent of what you said. On the very first slide, you showed us your three monoclonals to A1 only immunoprecipitated about 50 percent of 1.125 HDL and you briefly said something about a monoclonal to A2. You said that precipitated almost completely 1.125?

DR. CURTISS: No. It never got to 100 percent, obviously because there are particles that have A1 that don't have A2, but in that particular experiment we got to over 90 percent of HDL.

DR. GINSBERG: Which is much more than you'd expect though from most of the data that Dr. Cheung showed us.

DR. ALBERS: If you go back to the paper in 1971, that is entirely consistent, that observation.

DR. SCANU: (Inaudible question.)

DR. CURTISS: Oh, certainly not. You could see by the gels that there are other changes going on. Also, it is very important to point out that I use sodium hydroxide treatment as a model of aging and it is not a complete explanation. Certainly there are other things going on with respect to aging, but it does show that one needs to be very careful about the type of assays that one uses to quantitate HDL and be aware of the changes that could be observed. That is the key.

DR. INNERARITY: Dr. Curtiss, were the first group of antibodies made against A1 or HDL?

DR. CURTISS: They represented both. They were made against isolated ApoA1 that was isolated and stored and then used repeatedly as an immunogen and then, secondly, one of them was made against native HDL. At that point I was looking for antibodies that would distinguish allotypes. So, I isolated the HDL from a single patient, put it in a cold room and stored it under sterile conditions over the period of immunization which lasted up to three or four months. So, it was aged HDL that was used as an immunogen.

SPEAKER: Does the conformation of the apoprotein whether it was in a lipoprotein particle or whether it was delipidated make a difference?

DR. CURTISS: Certainly with those earlier antibodies we showed that the affinity of those antibodies for isolated A1 and HDL in some of them is identical, absolutely identical, and in other cases is not. They see the isolated protein much differently than the protein on a lipoprotein particle. This is going to be an individual characteristic of the particular monoclonal.

DR. ALBERS: Is there a difference in reactivity between A1/3 and A1/4 isolated from fresh HDL?

DR. CURTISS: I've not tested that.

SPEAKER: Did you test any other effects like temperature elevation or anything else besides sodium hydroxide?

DR. CURTISS: No, I haven't, Marcel's group in Montreal is doing a lot of that work and has published a portion of it and have shown that alkaline pH's, elevated storage temperatures, particularly four degrees versus freezing, the absence of anti-oxidants, all of these will contribute to changes in reactivity for some of his monoclonal antibodies. So, one needs to be very much aware of a number of different conditions in addition to this.

DR. HAVEL: Dr. Curtiss, in your response to Dr. Innerarity, you said you just left it in the cold room I assume at neutral pH at for degrees. Is there evidence that when you store that way that you de-amidate A1?

DR. CURTISS: I have not done those experiments, but those experiments have been done and, yes, de-amidation has been demonstrated and the release of ammonium has been demonstrated and sequence analysis has shown that that is one process that occurs. It is not all certainly, but it does occur with storage. I'd like Dr. Brewer to respond in terms of the occurrence of de-amidation in vivo and in vitro.

DR. BREWER: Well, I think it does occur. You can get one of the Als to change its isoform one part of which may in fact be de-amidation and that will occur at four degrees as you stored your sample. If you do serial IEFs, you can see the gradual

increase in the forms of those. Whether all of the A1, 3, and 4 is due only to de-amidation, as Dr. Havel is mentioning, that has been shown yet. That may be one thing that happens, but with 5 molar hydroxylamine, you can liberate a lot of different groups on A1 an you can have a lot of other changes in the structure which may influence it. De-amidation is one, but I'm not sure it is the only one.

DR. CURTISS: I would certainly agree with that.

DR. BREWER: In vivo, we have never seen any effect in the catabolism of A1, 3, 4 and 5. In all the studies that we've done, we've never seen any significant effect on the metabolic changes with what is occurring during the natural shift in those isoforms, assuming that that is in vivo de-amidation when those have been re-isolated. When you take the isolated A1, 3 4 and 5 isoforms from A1, it will just depend on your antibodies. In freshly isolated A1 that had those isoforms, whether they would be the same as the A1 isoforms that you get from storage, a direct comparison has not been made yet.

DR. SCANU: There are a number of proteolytic enzymes that should be discussed and demonstrated to be associated with HDL, in particular the amidation reaction. Even at four degrees, not everything is chemical or proteolytic in nature by simply de-amidation.

DR. ALBERS: I agree. Anything we do in terms of storage of HDL, we have to be concerned not only with the de-amidation, but also with oxidation and proteolytic effect.

DR. KRUL: Dr. Curtiss, I presume that most of your studies were done with HDL. Did you ever look at HDL subspecies isolated by density gradient or by other techniques to see whether or not it changed differently on different HDL subspecies?

DR. CURTISS: Not with the new antibodies. The work was published on the old antibodies, but not with the new antibodies.

SPEAKER: Dr. Oram, it was shown by many people that cholesterol can easily be stored in the membrane and movement between membrane of cholesterol can occur in the aqueous phase. In your system how do you know that the cholesterol movement through HDL requires binding? You are showing binding and cholesterol transfer to HDL, but how do you know that the two are associated with each other at all?

DR. ORAM: We don't believe in our system that binding is an absolute requirement for movement of cholesterol between cells and HDL. We think it is facilitated by binding based on studies from multiple directions where we can modify HDL proteins by various techniques, decrease binding and decrease cholesterol efflux and we can see clear associations between binding and efflux. What is missing obviously is to be able to modify the cell surface and specifically destroy binding and then see if it affects efflux or having some sort of mutant strain that doesn't have any receptor activity and see if binding and efflux are involved.

SPEAKER: You could treat the surface with trypsin.

DR. ORAM: If you treat the surface with trypsin, cholesterol transport is disrupted very badly and all the agents we have tried so far have had independent effects on membrane integrity and the cholesterol. So, we are still looking at that angle.

DR. SEGREST: Since the HDL3 particle was the one that seemed to affect the ACAT the most, how would you explain the studies in HDL3 and 2 as relative risk factors?

DR. ORAM: It would again depend on whether or not you look at them as precursor product-type relationships. Again, you have high HDL2 levels and if they are products of this kind of a transport system it may just be a reflection that it is going well, that you are clearing cholesterol from cells at a very efficient rate.

DR. SEGREST: Along the lines of my previous statement about post-prandial responses versus cholesteryl ester transport, I was interested in your statement that endothelial cells seem to have the highest number of receptors. Is that right? I could suggest another possibility for these receptors. That is that post-prandial lipolysis occurs at the endothelial cell surface and if there are particles produced that are abnormal it might be a nice place to have the HDL bound.

SPEAKER: You're making inferences now about influx of cholesterol in the HDL. So you have evidence that there is a binding-dependent component of influx? If you inhibit binding, are you also inhibiting influx?

DR. ORAM: The only inference I'm making is assuming that in net transport there is a difference between influx and efflux and that we're looking at efflux. So we have not done the type of experiments that you do which are obviously important experiments to look at that.

SPEAKER: I would expect both rates to be affected.

DR. ORAM: We see them when we destroy T and M. We see the net transport and efflux, both components do decrease which suggests that both components are dependent on binding, at least if you believe that the effect of T and M is related to its effect on binding.

DR. HAVEL: You refer to some experiments with proteoliposomes, but you didn't explain those. I assume that relates to measurements of efflux and transport. Can you enlarge on that?

DR. ORAM: Yes. If you prepare proteoliposomes and we haven't done the different subfractions--well, we've done E, A1 and A2 similar to the binding and we see the same kind of separation. Otherwise, A1 and A2 particles promote the best cholesterol efflux from cells and particles that contain E or no lipoprotein have less ability to promote cholesterol efflux from cells, consistent with what we know about binding. Now, of course there is a bigger background with these particles than we find with native particles.

DR. HAVEL: How about net transport?

DR. ORAM: Net transport, the same thing. I have not looked at E-containing particles yet. I have looked at A1 particles and the protein-free particles. When you compare those two, net transport occurs in the presence of vesicles that contain A1 but not in the absence of A1.

SPEAKER: Since both A1 and A2 containing vesicles effectively promote cholesterol efflux, have you examined what sites on A1 and A2 are shared? Do you have any ideas on that?

DR. ORAM: No, I don't. In fact, that's an excellent question because we haven't been able to find anything based on the amino acid composition and hydrophobicity or anything that would explain that kind of specificity. So, if somebody has some suggestions, I'd be happy to hear about them. We don't know what kind of signal it could be at this stage.

DR. MELCHIOR: Dr. Oram, are you suggesting that all cholesterol efflux in cells, 100 percent of whatever you see in the system is being mediated by a binding site, a receptor binding site? You can get significant efflux from liposomes, bile acid micelles, or from red blood cells that don't have binding sites.

DR. ORAM: No, we don't think it is. In fact, we think that HDL facilitates movement of cholesterol from cells, probably an acute response to an overload in the cell and that during steady state conditions you don't need binding to get efflux. I would not suggest that at all. But we think there may be some targeting mechanisms going on where HDL is actually moving cholesterol from specific pools in the cell and pools that may be related to the substrate for the ACAT enzyme and why we see these big changes in ACAT activity.

DR. MELCHIOR: So you say that under most conditions that mass of cholesterol that comes out, if you're getting a mass release of cholesterol, is not coming out through a receptor-mediated-process.

DR. ORAM: No, that's not necessarily true. If you do saturation curves at low concentrations of HDL, I would say, if you are below saturation of receptor, mass movement of cholesterol from the cell to the particle, most of it is dependent on binding. However, at very high concentrations you have very non-specific or low affinity component of binding that is not dependent on receptor binding. So, under those conditions, I would say, yes, most of the mass movement of cholesterol from cells is not dependent on binding .

DR. SEGREST: Dr. Oram, since cholesterol-free liposomes remove cholesterol avidly from red blood cells, I'm a little surprised that in your system you didn't get net transport into those.

DR. ORAM: No, we do get them in the presence of apoprotein-free liposomes. We've seen it enhanced when they have apoproteins on them.

SESSION IX. Metabolism of High Density Lipoprotein

Chair: Paul Roheim

METABOLIC HETEROGENEITY OF HIGH DENSITY LIPOPROTEINS

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Introduction

The high density lipoprotein (HDL) fraction in human plasma is heterogeneous in terms of particle size, density and apolipoprotein composition; it is also heterogeneous in terms of metabolic function. This paper is concerned with HDL particle size, with the factors that regulate it and with its physiological significance. Particular attention will be given to the metabolic implications of subpopulations of very small HDL particles. It will be argued that these small particles are highly reactive and that their physiological importance may be much greater than implied by their normally very low concentration in plasma.

HDL subpopulations based on differences in particle size

Polyacrylamide gradient gel electrophoresis has been used to separate human HDL into a number of subpopulations of differing particle size. The high resolution of this technique relies on the migration of charged intact lipoproteins through a matrix of progressively decreasing pore size. Since migration of the charged particles is limited as they approach an exclusion limit, the distance of migration through the gel is a function of the size and shape of the particles.

Anderson et al (1) subjected human HDL to gradient gel electrophoresis and reported the existence of at least three particle size ranges, corresponding to the HDL_{2a}, HDL_{2b} and HDL_3 fractions isolated ultracentrifugally in the density intervals 1.063-1.100 g/ml, 1.100-1.125 g/ml and 1.125-1.21 g/ml, respectively. The size of particles comprising these populations was corroborated by electron microscopy.

Gradient gel electrophoresis was also used by Nichols et al (2) and Blanche et al (3) to demonstrate further heterogeneity within the HDL_3 subclass. In total, five subpopulations of HDL were identified in human plasma. The two subpopulations containing the largest particles, designated HDL_{2b} and HDL_{2a}, corresponded to the ultracentrifugal fractions described above. The three populations of smaller particles, HDL_{3a}, HDL_{3b} and HDL_{3c}, were all subpopulations within the ultracentrifugal HDL_3 subclass. HDL_{3a} and HDL_{3b} corresponded to subpopulations characterized by rate-zonal ultracentrifugation and termed HDL_{3L} and HDL_{3D} (4). Analysis of the frequency distribution of different sized HDL particles in 194 human subjects showed that these five subpopulations consisted of particles with mean radii of 5.3 nm, 4.6 nm, 4.2 nm, 4.0 nm and 3.8 nm, respectively (3).

Variation in the concentration of HDL subpopulations

The concentration of the total HDL fraction in human plasma varies from subject to subject. This variation, however, is a reflection of

differences in concentration of the constitutive subpopulations. For example, the higher concentration of HDL in female compared to male subjects results from an increased concentration of HDL_2 subpopulations (5). Similar conclusions were drawn from differences in floatation rates of HDL subclasses during analytical ultracentrifugation of HDL from males and females (6). Conversely, the lower than normal concentration of HDL in hypertriglyceridemic subjects (5,7) is largely a reflection of a reduced concentration of subpopulations within the ultracentrifugal HDL_2 subclass.

Hypertriglyceridemia has also been shown to influence the distribution of subpopulations within the ultracentrifugal HDL_3 subclass. Patsch et al (4) found that HDL_{3L}, identified as a less dense fraction by zonal ultracentrifugation, was the predominant subpopulation of HDL_3 in normotriglyceridemic subjects. On the other hand, some evidence was obtained that the low concentration of HDL in the plasma of hypertriglyceridemic subjects was associated with the presence of a more dense fraction, HDL_{3D}, and that in some subjects this subpopulation accounted for the whole of the HDL fraction (7). Similar preliminary findings for the HDL_{3a} and HDL_{3b} subpopulations have also been reported (8). We have recently used gradient gel electrophoresis to study the particle size distribution of HDL in subjects with a wide range of plasma triglyceride concentrations (9). The relative deficiency of HDL_2 in subjects with hypertriglyceridemia was confirmed. It was also shown that the mean particle size of HDL_3 decreased progressively as the plasma triglyceride concentration increased. Subjects with the highest plasma triglyceride concentrations had an HDL fraction consisting almost entirely of very small particles, the size of which was on the boundary between HDL_{3b} and HDL_{3c}.

The interrelationships between different subpopulations of HDL are still poorly understood, although there is evidence that at least some are interconvertible (10,11). Several plasma factors are known to influence the particle size and density of human HDL during incubation in vitro. Some factors increase, while others decrease the particle size of HDL. Thus, the HDL subpopulation distribution in vivo is likely to reflect a balance between opposing forces.

Plasma factors which influence the particle size of HDL

Lipoprotein lipase. Lipoprotein lipase acts primarily to hydrolyze triglyceride in chylomicrons and VLDL. The resulting reduction in volume of the lipoprotein core creates a surplus of surface constituents which, in vitro, transfer to the HDL fraction. This is associated with a reduction in the density of HDL_3 towards that of HDL_2 (12,13). Indeed, the concentration of HDL_2 in vivo correlates positively with activity of lipoprotein lipase (14) and the low concentration of HDL_2 which accompanies hypertriglyceridemia has been attributed to a reduced supply of surface constituents resulting from a diminished lipolysis of triglyceride-rich lipoproteins. It should be noted, however, that this mechanism does not explain why the concentration of HDL_2 is also low in subjects in whom hypertriglyceridemia is a consequence of overproduction rather than decreased lipolysis. It should also be noted that while activity of lipoprotein lipase may provide surface constituents to an expanding HDL particle, there must be an additional mechanism to account for the increased concentration of cholesteryl esters in HDL_2.

Lecithin:cholesterol acyltransferase (LCAT). LCAT catalyses the esterification of plasma cholesterol and is thus responsible for increasing the cholesteryl ester content and, by implication, the particle size of plasma lipoproteins. The effect of LCAT on the size and density of HDL, however, has not been clearly defined. Some reports have concluded that LCAT is of major importance in the conversion of small to larger HDL particles (15-17), while others have suggested that LCAT alone has little effect on HDL particle size or density (18). Studies using purified enzyme have shown that LCAT can convert very small spherical HDL (19) or discoidal complexes of apo AI, phosphatidylcholine and cholesterol (20,21) into particles resembling normal spherical HDL_3. By contrast, most studies seeking to demonstrate a role of LCAT in the conversion of HDL_3 to HDL_2 have either used only partially pure preparations of the enzyme or have inferred an involvement by the use of chemical inhibitors that may have affected more than LCAT (11,16). We have recently addressed this issue by examining the effects of purified LCAT on the particle size of HDL_{3a} and HDL_{3b} during incubation in vitro (22).

Subpopulations of HDL were isolated by a combination of ultracentrifugation and gel permeation chromatography and incubated at 37°C with a mixture containing purified LCAT, human serum albumin and varying concentrations of human LDL (Fig. 1). Incubation of HDL_{3a} (mean particle radius 4.3 nm) in the absence of LDL resulted in an esterification of more than 70% of the HDL free cholesterol after 24 hours of incubation. However, this was sufficient to increase the HDL cholesteryl ester content by less than 10% and was accompanied by only minimal changes in HDL particle size. When this same mixture was incubated in the presence of progressively increasing concentrations of LDL as a donor of free cholesterol, the molar rate of production of cholesteryl ester was much greater. At the highest LDL concentration the HDL cholesteryl ester content was almost doubled after incubation for 24 hours and the size of the particles was increased to that of HDL_2 (Fig. 1). Similar results were obtained in the case of HDL_{3b} (mean radius 3.9 nm) (Fig. 1). Thus, the capacity of purified LCAT to increase the size of normal HDL_3 appears to be related to the amount of cholesteryl ester formed which in turn relates to the presence of a donor (such as LDL) of free cholesterol and possibly phospholipid for the reaction.

It should be noted that the HDL_2-like particles formed in vitro by LCAT differ in several ways from native HDL_2 (22). They are overly enriched with cholesteryl esters and relatively depleted of phospholipid. Furthermore, the ratio of apo AI:apo AII in the enlarged particles resembles that in the original HDL_3 and is much lower than in native HDL_2. Thus, while LCAT is obviously an important factor in the formation of larger HDL, it is also apparent that, in vivo, other factors must modulate the composition of these particles towards that of native HDL_2.

An effect of LCAT on HDL morphology in vivo may be inferred by an examination of human subjects who are deficient in activity of the enzyme. The HDL fraction in these subjects consists of a mixture of discs and spheres (23), both of which are markedly deficient in cholesteryl esters. The spherical particles are very small, equating in size with HDL_{3c} (23). When plasma from subjects with LCAT deficiency is supplemented with exogenous LCAT and incubated in vitro,

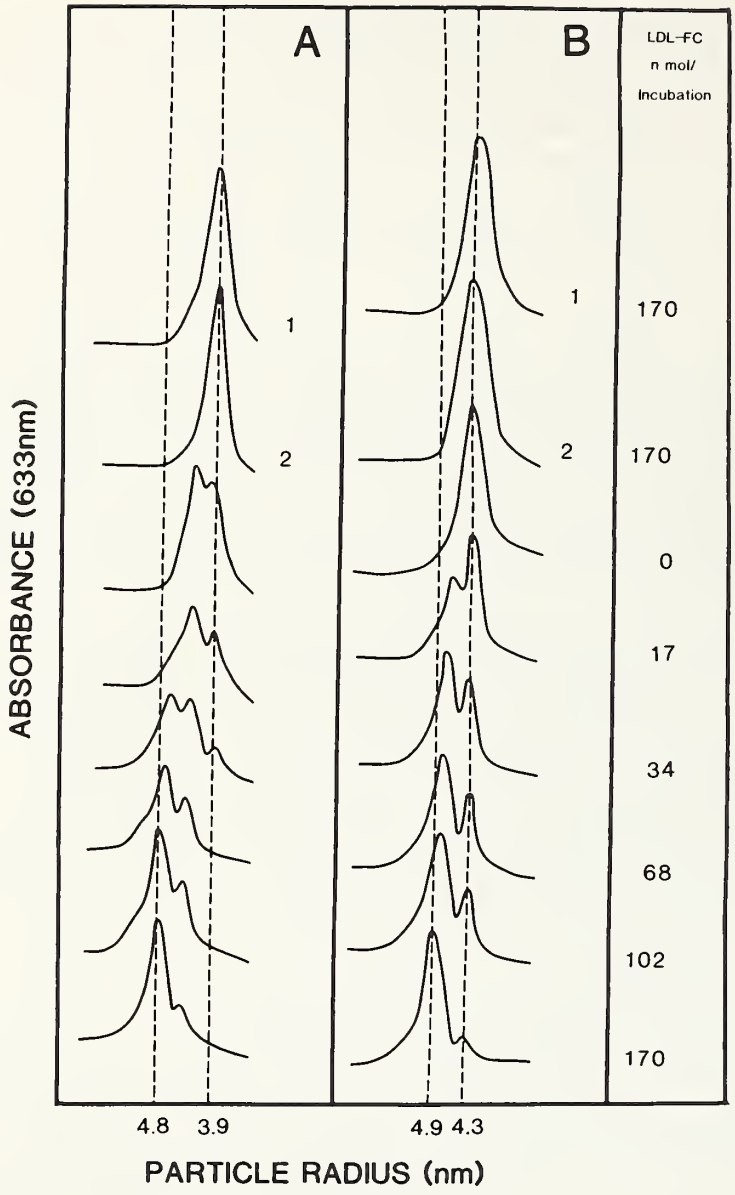

Figure 1. Effects of LCAT on the particle size of HDL_3: consequences of varying the concentration of LDL. The tracings represent gradient gel electrophoretic profiles of HDL (22). Preparations of HDL_{3b} of mean particle radius 3.9 nm (free cholesterol 7.5 nmol) or HDL_{3a} of mean particle radius 4.3 nm (free cholesterol 10 nmol) were mixed with purified LCAT (25 units) and varying amounts of LDL (providing free cholesterol ranging from 0 to 170 nmol) and incubated at 37°C for 24 h. Human serum albumin was present at a final concentration of 37 mg/ml in all mixtures. The volume of all incubation mixtures was made to 0.25 ml by the addition (if necessary) of Tris.HCl buffer. The upper patterns (shown on each panel) are the non-incubated 4°C controls (Tracing 1) and the controls incubated at 37°C for 24 h in the absence of LCAT (Tracing 2). For HDL_{3b} (panel A) the amounts of free cholesterol esterified during the 24 h incubation at the lowest and highest concentration of LDL were 7 and 90 nmol respectively. The corresponding rates of esterification in the studies with HDL_{3a} (panel B) were 9 and 99 nmol respectively.

both the discs and the small spherical HDL acquire cholesteryl esters and are converted into larger spherical HDL (23).

Lipid transfer protein (LTP). When human HDL are incubated in vitro with triglyceride-rich lipoproteins and a source of LTP there is an appearance of both smaller and larger HDL particles (24). The mechanism of formation of the small particles and their relationship to the lipid transfer process is uncertain. The formation of larger HDL, however, can be readily explained in terms of the transfer process which results in a partial replacement of HDL cholesteryl esters by triglyceride (25,26). Since triglyceride has a molecular volume which is greater than that of cholesteryl ester (27), this change in composition is associated with an appearance of a population of enlarged HDL particles (24).

The formation of large, triglyceride-rich HDL in vitro, however, contrasts markedly with the situation in vivo. Human subjects with hypertriglyceridemia, in whom there is likely to be an enhanced rate of lipid transfer between HDL and triglyceride-rich lipoproteins in vivo, have HDL particles that are smaller, not larger than normal (9). Conversely, rat plasma is almost totally deficient in LTP activity (28) and contains HDL particles that are considerably larger than those in human plasma (29). Furthermore, we have shown that injection of human LTP into rats is associated in vivo with a reduction in HDL particle size (29). Thus, the finding that HDL particle size is increased by neutral lipid transfers in vitro is not consistent with a number of observations in vivo.

In recent studies of rabbits, however, we have found that the situation in vivo does mimic what takes place during incubations in vitro. The HDL fraction in rabbit plasma consists of large, triglyceride-rich, cholesteryl ester depleted particles (30), comparable to those formed in vitro by incubating human HDL with LTP and Intralipid (as a model of a triglyceride-rich lipoprotein) (24). Furthermore, intravenous infusion of Intralipid into rabbits is associated with an appearance in vivo of HDL that are even larger (Fig. 2A) and even more enriched with triglyceride. An identical result is observed in vitro when rabbit plasma is supplemented with Intralipid (Fig. 2B). Thus, by contrast with the situation in studies of human and rat plasma, in rabbits there is a consistency between what is observed in vivo and in vitro. This species difference may relate to the fact that rabbit plasma is a rich source of LTP activity (28) but is deficient in activity of hepatic lipase (31).

Hepatic lipase. Hepatic triglyceride lipase hydrolyses phospholipid and triglyceride in HDL (32) and has been reported to promote an increase in the density of HDL particles (33). In the absence of activity of hepatic lipase, it would be predicted that triglyceride transferred into HDL will not be hydrolysed and will accumulate in progressively enlarging particles. Thus, the finding that activity of hepatic lipase in post-heparin rabbit plasma was less than 5% of that in comparable preparations of rat plasma (unpublished observation), provided a logical explanation for both the large particle size and the triglyceride enrichment of rabbit HDL.

When rabbit plasma was supplemented with hepatic lipase isolated from post-heparin rat plasma and incubated in vitro, there was a marked

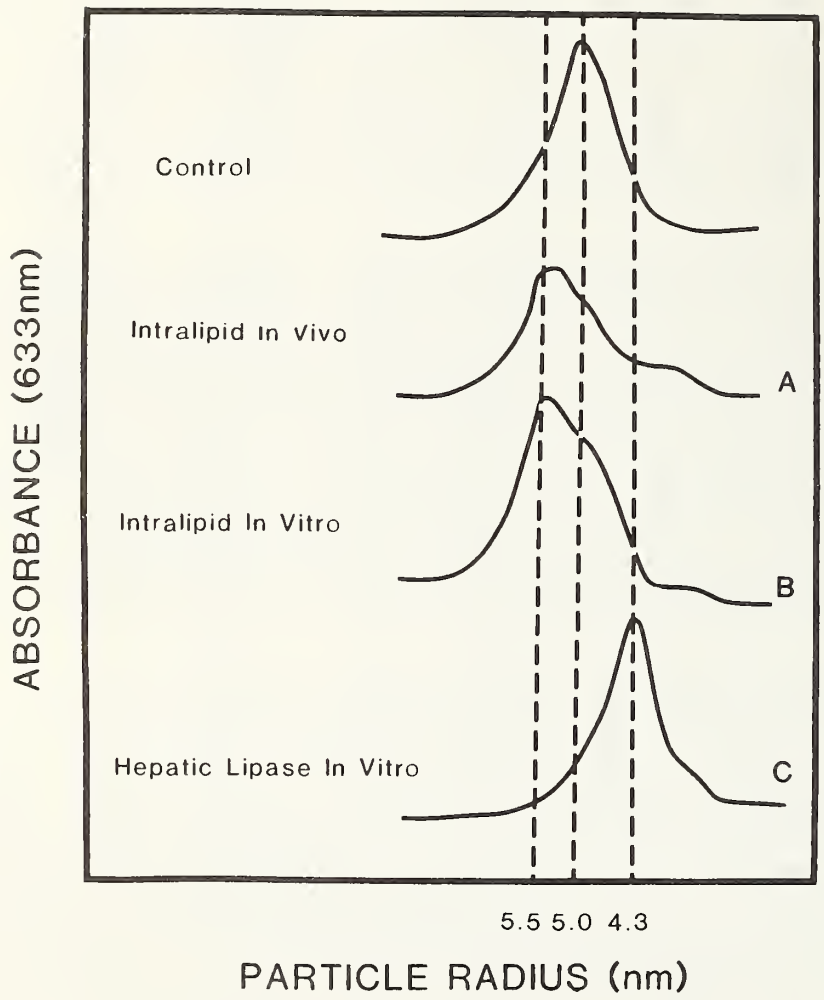

Figure 2. Effects of Intralipid and hepatic lipase on the particle size of rabbit HDL. The control tracing shows the gradient gel electrophoretic profile of HDL in untreated rabbit plasma. Tracing A shows the HDL profile after the rabbit had been infused intravenously for 4 hours (2 ml per hour) with 20% Intralipid. Tracing B shows the HDL profile after control rabbit plasma had been incubated in vitro for 4 hours at 37°C in the presence of Intralipid (15% v/v). Tracing C shows the HDL profile after control rabbit plasma had been incubated in vitro for 4 hours at 37°C in the presence of rat hepatic lipase (15% U/ml). Activity of LCAT was inhibited in all incubation mixtures by the presence of 0.002 M parachlormercuriphenyl sulfonate.

reduction in the triglyceride content of HDL and a change in overall composition towards that of human HDL_3. Concurrently, the rabbit HDL fraction was converted from particles of a size similar to that of human HDL_2 to particles the same size as human HDL_3 (Fig. 2C). It was postulated, therefore, that the enlarged triglyceride-rich particles formed in vitro during incubation of human HDL in the presence of Intralipid and LTP, were a consequence of the absence of hepatic lipase activity. Conversely, the fact that enlarged, triglyceride-rich HDL to not accumulate in the plasma of subjects with hypertriglyceridemia may reflect the presence of hepatic lipase activity in vivo. We have tested this proposition in vitro in studies of HDL_3 isolated from normal human subjects.

Initially, human HDL_3 were incubated with Intralipid and a source of LTP as in earlier studies (24). The enlarged triglyceride-rich HDL formed during the incubation were recovered and subjected to a second incubation in the presence of rat hepatic lipase (34). Activity of hepatic lipase resulted in a marked reduction in particle size to form HDL which were even smaller and more depleted of core content than those observed in vivo in subjects with hypertriglyceridemia. In subsequent experiments, the combined effects of lipid transfers and hepatic lipase on HDL particles were studied when both processes were operating simultaneously. A preparation of human HDL_3 was incubated with a mixture of LTP, Intralipid and hepatic lipase. An initial population of particles of mean radius 4.3 nm (HDL_{3a}), the predominant subpopulation of HDL_3 in normal subjects, was converted into populations of smaller particles of a size equating with HDL_{3b} and HDL_{3c} (Fig. 3) (34). These smaller particles predominate in the plasma of subjects with hypertriglyceridemia (9).

HDL conversion protein

It has been reported recently that changes in particle size and density of HDL may occur in vitro when there is no activity of LCAT, LTP or either of the lipases. This observation led to the postulation that such changes in HDL particle size are due to a previously unidentified factor (10,35,36). A protein possessing such activity and designated HDL conversion protein, has recently been isolated by subjecting human plasma sequentially to: precipitation with ammonium sulfate (35%-50% saturation), ultracentrifugation to recover the fraction of density 1.21-1.25 g/ml, cation exchange chromatography, gel permeation chromatography and anion exchange chromatography (unpublished observation). These steps resulted in the purification of a protein which on SDS polyacrylamide gel electrophoresis appeared as a single band with an apparent M_r of 58,000. This preparation promoted dramatic changes in the particle size of human HDL_3 during incubation in vitro. It did not, however, promote cholesterol esterification, lipid transfers or lipolysis.

Addition of the conversion protein to a relatively homogeneous population of human HDL_{3a} (particle radius 4.3 nm) promoted the formation of a series of new populations of particles, some as small as HDL_{3c} (radius 3.8 nm) and others well into the HDL_2 range (Fig. 4). The conversion process was both time dependent and dependent on concentration of the conversion protein. The HDL products of the conversion have still not been completely characterized, although preliminary

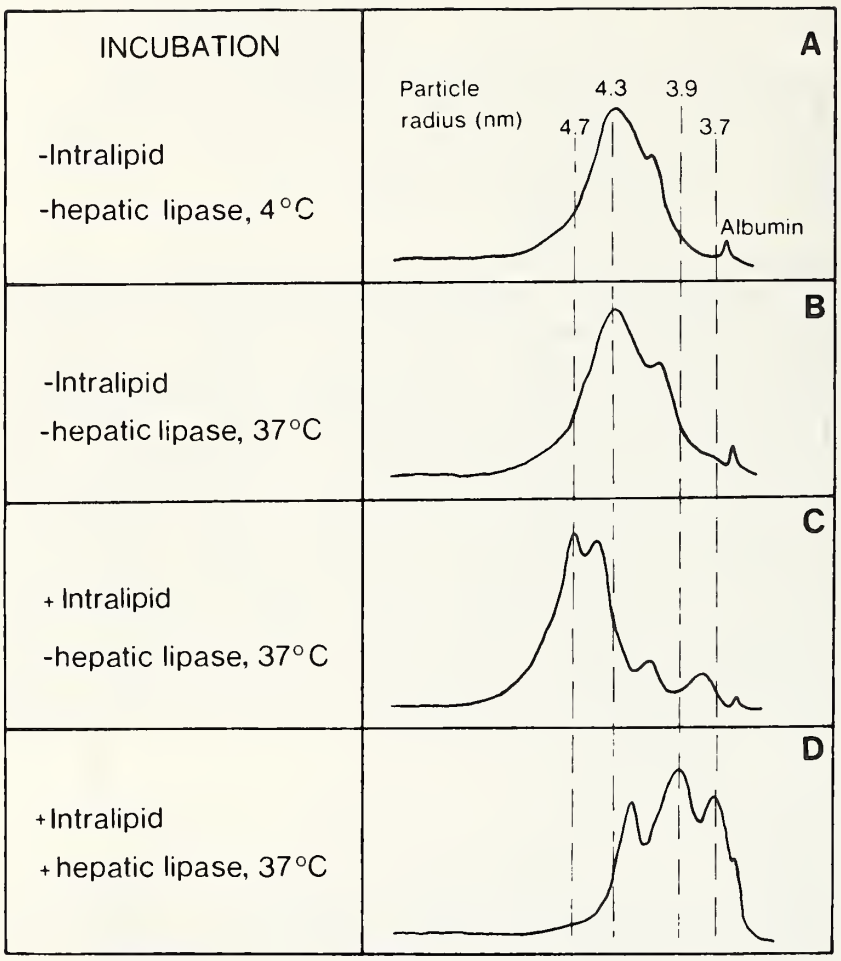

Figure 3. Incubation of human HDL_3 in the presence of a mixture of Intralipid, a source of lipid transfer protein and hepatic lipase. HDL_3 (d 1.13-1.21 g/ml) were either kept at 4°C (panel A) or incubated at 37°C for 24 hours (panel B) in the absence of both Intralipid and hepatic lipase. Aliquots of the same preparation of HDL_3 were also incubated at 37°C for 24 hours in the presence of Intralipid (15% v/v), but in the absence of hepatic lipase, (panel C) or in the presence of both Intralipid (15% v/v) and hepatic lipase (15.6 U/ml) (panel D). All incubations also contained heparin (70 U/ml) and lipoprotein-free plasma which had previously been heated to inactivate LCAT. Following incubation, the samples were subjected to ultracentrifugation to remove albumin and then analyzed by gradient gel electrophoresis.

studies have indicated that the larger conversion products have a lipid and total protein content similar to native HDL_2 (36). The ratio of apo AI:apo AII in these particles, however, was lower than in the original HDL_3 and very much lower than in native HDL_2. Compared to the parent HDL_3, the smaller conversion products (equating in size to HDL_{3C}) were depleted of cholesteryl ester and apo AII (36).

Influence of apolipoprotein composition on HDL particle size

An indication that apolipoprotein composition may have a dramatic effect on HDL size and density was provided recently in studies of HDL enriched with the acute phase reactant, serum amyloid A protein (SAA) (37). We isolated SAA-enriched HDL from human subjects with myocardial infarction and from a subject with secondary amyloidosis. SAA enrichment was also achieved in vitro by adding the protein to incubations of normal HDL. Regardless of the origin of the SAA enrichment, the modified HDL had a density equivalent to HDL_3, but differed in composition (37). When compared to normal HDL_3 the SAA-enriched HDL had a reduced phospholipid and an increased triglyceride content (37). Furthermore, when separated on the basis of size by gradient gel electrophoresis, the SAA-enriched HDL were much larger than normal HDL_3 and, with a radius of 4.5-5.3 nm, extended well into the HDL_2 size range. The size of the enriched particles also correlated positively with SAA content. The functional implications of these changes in HDL size and composition, however, are not yet apparent.

Relationship between HDL particle size and reactivity with LCAT

The capacity of different preparations of HDL to act as substrates for the cholesterol esterifying action of LCAT is known to vary with particle size. The HDL_3 subfraction, for example, was shown many years ago to be preferred over HDL_2 (38). In fact, HDL_2 was reported to inhibit cholesterol esterification (39-41), although in more recent studies we have shown that this may only reflect competition for LCAT by a less reactive substrate (42).

An inverse relationship between particle size and substrate reactivity with LCAT has also been found to apply to different subpopulations within the HDL_3 subfraction (43). The explanation for this enhanced substrate reactivity of smaller HDL_3 particles is uncertain. Factors such as the ratio of phospholipid to free cholesterol and the ratio of apo AI to apo AII which have been reported to influence the reactivity of artificial substrates (44,45), have been found not to correlate with the substrate reactivity of different preparations of native HDL (46). It has been postulated that the increased content of cholesteryl esters in larger particles may exert a feedback inhibition on LCAT (44). Indeed, in studies in which the cholesteryl ester content of HDL_3 was reduced in vitro by transfer to triglyceride-rich particles, the LCAT reactivity of the HDL was enhanced (47). It was also found, however, that coincident with a depletion of cholesteryl ester, there were changes in lipoprotein particle size (47). Subsequent studies have indicated that this enhanced reactivity with LCAT relates more to a reduction in particle size than to a depletion of cholesteryl ester content (43).

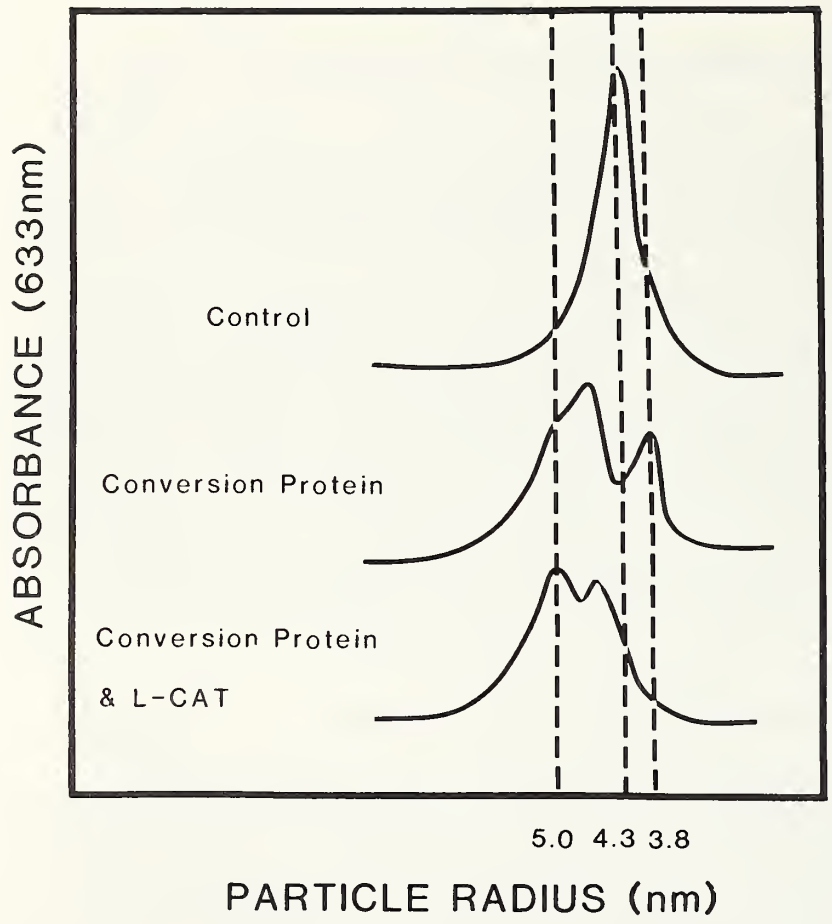

Figure 4. Gradient gel electrophoresis of human HDL_{3a} following incubation with HDL conversion protein and LCAT. A preparation of HDL_{3a} of mean particle radius 4.3 nm (free cholesterol 11 nmol) was mixed with either conversion protein (20 μg) alone or conversion protein and LCAT (35 units) and incubated at 37°C for 24 hours. Other details are as given in the legend to Figure 1. The control sample represents the gradient gel electrophoretic profile of HDL_{3a} maintained at 4°C. An incubated control containing Tris.HCl buffer in place of conversion protein and LCAT also gave an identical pattern (not shown in the figure).

Preparations of human HDL_{3a} (mean particle radius 4.3 nm) have been compared with HDL_{3b} (mean radius 3.9 nm) as substrates for LCAT. The Vmax of the cholesterol esterification reaction with HDL_{3b} as substrate was about double that with HDL_{3a} (43). This observation is consistent with the much greater rate of cholesterol esterification in the plasma of human subjects with hypertriglyceridemia (48) in whom HDL_{3b} is the major subpopulation (9). Indeed, it has been demonstrated that HDL_3 from hypertriglyceridemic subjects has a considerably greater capacity to react with LCAT than HDL_3 from normal subjects (43). There have been no reports of comparable studies with HDL_{3c}, although these small particles would be predicted to be highly reactive with LCAT.

HDL_{3c} are normally a very minor component of the HDL fraction (3) and are frequently not identifiable. This is in apparent conflict with the ease with which they are formed during incubation in vitro (see above). However, as discussed earlier, the particle size of HDL in vivo represents a balance between a number of opposing forces. Given that HDL_{3c} are likely to be especially reactive with LCAT and given that one consequence of such an interaction will be an increase in the particle size, it is probable that, in vivo, a conversion of HDL_{3c} into larger particles may be extremely rapid. Very small HDL may therefore exist only transiently in vivo in normal human plasma.

We have recently performed preliminary studies to investigate the capacity of LCAT to prevent an accumulation of very small HDL particles in vitro. Incubation of HDL_{3a} with a preparation of purified conversion protein resulted in the formation of populations of HDL equating in size with HDL_{2a} and HDL_{3c} (Fig. 4). When LCAT was included in the incubation mixture the HDL_{3c} were no longer apparent; in their place were particles the size of HDL_{2b} (Fig. 4).

Metabolic significance of small HDL particles

As outlined above, very small HDL particles are formed in vitro by two quite different mechanisms: (i) larger HDL are reduced in size as a result of the replacement of cholesteryl esters by triglyceride with subsequent hydrolysis of the triglyceride by hepatic lipase and (ii) as one of the products of activity of the HDL conversion protein. Obvious questions arise: do these in vitro phenomena have in vivo correlates? If so, what are the metabolic implications? These issues will be discussed by considering each of the mechanisms separately.

Small HDL produced by lipid transfers and hepatic lipase. This mechanism almost certainly accounts for the small particle size of HDL in subjects with hypertriglyceridemia. One consequence of the presence of these smaller particles is their increased reactivity with LCAT. Indeed, as discussed above, the elevated rate of plasma cholesterol esterification in subjects with hypertriglyceridemia (48) has been shown to relate at least partly to the enhanced capacity of the HDL from such subjects to act as substrates for LCAT (43). Thus, both the rate of plasma cholesterol esterification and the rate of transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins increase with increasing concentrations of plasma triglyceride. It follows, therefore, that the rate of entry of free cholesterol into the plasma and the rate of exit of cholesteryl esters from the plasma must also

increase with increasing plasma triglyceride concentration. This raises an interesting question in relation to the mechanism by which HDL "protects" against the development of coronary heart disease.

One view holds that elevated concentrations of HDL "protect" by enhancing reverse cholesterol transport and thus increasing the rate of efflux of cholesterol from the arterial wall. Cholesterol moves from tissues into the circulation by diffusion down a concentration gradient maintained by the esterification of cholesterol on the surface of plasma HDL. The rate of cholesterol esterification, therefore, provides one measure of reverse cholesterol transport. If the proposition is correct that HDL "protect" by enhancing reverse cholesterol transport, it follows that the rate of plasma cholesterol esterification should correlate positively with the concentration of HDL. In fact, quite the reverse is true: when the concentration (and particle size) of HDL are reduced, the rate of plasma cholesterol esterification is increased. If, therefore, a "protective" role of HDL is not explicable in terms of an increased rate of reverse cholesterol transport, it is worth briefly considering alternate mechanisms underlying the well documented inverse correlation between the concentration of HDL and the development of coronary heart disease. We propose two alternate mechanisms, each consistent with experimental observations.

(i) Instead of assuming that a high concentration of HDL is protective, it is possible that a low concentration is harmful as a direct consequence of the presence of the small HDL particles that predominate under such conditions. Since these small particles are highly reactive with LCAT, the rate of plasma cholesterol esterification is enhanced and the rate of entry of cholesterol into the plasma is increased. If the cholesteryl esters so formed were to remain with HDL, the particles would enlarge and the rate of esterification decrease. The existence of small particles in the presence of an enhanced rate of esterification indicates that the cholesteryl esters do not remain with the HDL, but rather that they are transferred to other (potentially atherogenic) lipoprotein fractions. The epidemiological observation that the concentration of HDL correlates inversely with the development of coronary heart disease may thus relate to the capacity of small HDL particles to generate an increased load of cholesteryl esters which reach the arterial wall as a component of other (atherogenic) lipoproteins.

(ii) Yet another mechanism by which HDL may appear to be protective is that the concentration of HDL may be a marker for some other atherogenic factor. Given the inverse correlation between both the concentration and the particle size of HDL and the concentration of triglyceride-rich lipoproteins (9), it would not be surprising to find a comparable correlation between HDL and the remnants of VLDL and chylomicrons. However, while such remnants may be a significant presence for most of the 24 hours, they may either be absent or not recognized in a plasma sample collected after a 12-14 hour fast. A possibility that both the concentration and the particle size of HDL correlate inversely with the 24-hour integrated concentration of atherogenic remnant particles has been neither tested nor excluded by any of the reported epidemiological studies.

Small HDL particles generated by activity of the HDL conversion protein. The small HDL particles formed in vitro by lipid transfers and activity of hepatic lipase have, as discussed above, an obvious

in vivo correlate in the small HDL observed in subjects with hypertriglyceridemia. The small products of the HDL conversion protein, by contrast, do not appear to equate with particles observed in freshly collected plasma. In vitro, activity of the conversion protein promotes a significant formation of very small HDL in human plasma after 12-24 hours of incubation (36). Given that human HDL turnover in vivo with a $T_{\frac{1}{2}}$ of 3-5 days (49), there is clearly sufficient time for the conversion protein to act and to generate small particles. As suggested above, however, it is possible that the small particles are especially reactive with LCAT and that they are reconverted into larger particles as fast as they are formed. If this is the case, their formation may play an important role in cholesterol homeostasis.

A population of very small, highly reactive HDL may provide the mechanism by which cholesterol is removed from cells that lie outside the vascular space. The continuing regeneration of such particles may also play a role in the regulation of plasma cholesterol esterification, especially under circumstances when transfers of cholesteryl esters to triglyceride-rich lipoproteins are minimal. The rat, a species deficient in LTP (28) but rich in activity of the HDL conversion protein (36), may provide a useful model in which to investigate the role of the conversion protein in the regulation of plasma cholesterol esterification.

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SOME UNIQUE PROPERTIES OF APO A-I-CONTAINING LIPOPROTEINS WITH PRE-BETA ELECTROPHORETIC MOBILITY

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Introduction

The human high density lipoproteins (HDL) are a heterogeneous group of lipoproteins that are thought to perform a wide variety of functions. These diverse functions include participation in reverse cholesterol transport (1), stimulation of vascular endothelial cell growth (2), participation in the coagulation process (3), inhibition of protozoal parasite growth (4), and delivery of cholesterol to steroidogenic cells (5-8). Presumably, only selected HDL subspecies can perform each of the functions. Assignment of specific functions to individual HDL subspecies first requires the identification and isolation of each subspecies.

While particle hetrogeneity within the HDL class has been well documented by ultracentrifugation (9-12), electrophoresis (13-17), and chromatography (18-23), the characterization of unique HDL subspecies has been a difficult process. The primary problems have been a) the inability to separate cleanly individual HDL subspecies and b) the possibility that methods employed for isolation may create structural artifacts.

An alternative method, which we have termed selected-affinity immunosorption (24), has been developed for the isolation of apparently native apolipoprotein (apo) A-I-containing lipoproteins. (Note: Technically, HDL are those lipoproteins with buoyant densities between 1.063 and 1.21 g/ml. Selected- affinity immunosorption using antibodies directed against apo A-I is used to isolate all lipoproteins containing apo A-I. HDL are functionally considered to be lipoproteins containing apo A-I. For the purpose of simplification, we will refer to the apo A-I-containing lipoproteins isolated by selected-affinity immunosorption as immunosorbed HDL.) In this method the HDL are isolated by elution from a pauciclonal immunoaffinity column with a preselected buffer that is minimally perturbing to the lipoproteins. These HDL are therefore a suitable pool from which to separate individual subspecies.

A unique apo A-I-containing lipoprotein with pre-beta electrophoretic mobility (pre-beta HDL) has been isolated from the pool of immunosorbed HDL by starch block electrophoresis (25). This lipoprotein was present in plasma and serum before isolation, and possesses properties different from the bulk of HDL. Presented here are our observations on these structural and functional differences.

Methods

Isolation of immunosorbed HDL. The immunosorbed HDL were isolated from plasma or serum by selected-affinity immunosorption, described in detail previously (24). Briefly, antibodies against apo A-I were isolated by binding them to an apo A-I-Sepharose column and eluting a subpopulation of antibodies that dissociated with an elution buffer selected to be minimally perturbing to HDL structure. The antibodies were themselves then cross-linked to Sepharose to form the anti-apo A-I-Sepharose column used to isolate the lipoproteins. Plasma or serum was applied to the anti-apo A-I-Sepharose column, and a 0.015 M Tris, pH 7.4, 0.15 M NaCl buffer was used to wash out the unretained protein. The buffer was then changed to 0.2 M acetic acid, pH 3.0 (the same buffer used to isolate the antibodies), and HDL were recovered. The eluate was immediately neutralized with 2.0 M Tris, concentrated to 2 mg of protein/ml by ultrafiltration with an Amicon YM-10 membrane under a pressure of 20 psi, and then dialyzed against 0.15 M NaCl that contained preservatives.

Isolation of immunosorbed HDL. Apo A-I-containing lipoproteins with pre-beta mobility were isolated from immunosorbed HDL by starch block electrophoresis (25). Potato starch (Granny Goose, Oakland, CA) was washed with distilled water and then suspended in 0.076 M barbital buffer, pH 8.6. The starch was poured into a 1 x 40 x 10 cm block. A concentrate of immunosorbed HDL (approximately 15 mg of protein) was applied in a zone 5 cm from the anodic end of the block and electrophoresed for 18 hr at a constant current of 80 ma. The block was divided into 1-cm segments along the direction of migration and the lipoproteins in each segment were recovered by two 3-ml washes of the starch with 0.15 M NaCl. The apolipoprotein distributions in the fractions were determined by immunonephelometry. The appropriate fractions were pooled for further analysis.

Characterization of pre-beta HDL. The chemical analyses of lipoproteins for their contents of protein, phospholipid, cholesterol, cholesteryl ester, and triglyceride were performed as previously described (24). Apoproteins were resolved by SDS gradient gel electrophoresis (5-25%) and detected by immunonephelometry (26) or immunodiffusion. Immunoelectrophoresis, used to detect pre-beta HDL, was performed by a modification (25) of the procedure of Grabor and Williams. Circular dichroic spectra of HDL were determined from 240 to 200 nm after precise measurements of protein concentration. Liquid phase competition for HDL binding to monoclonal antibodies was performed as described by Curtiss (27). Binding of HDL to membrane surfaces was performed as previously described (28).

Results and Discussion

In addition to classical HDL of alpha electrophoretic mobility there exist immunosorbed HDL with pre-beta electrophoretic mobility (pre-beta HDL). This HDL subspecies can be found in plasma as well as HDL isolated by selected-affinity immunosorption. It is most easily visualized by immunoelectrophoresis using antisera directed against apo A-I (Fig. 1, inset). Preparatively, pre-beta HDL can be isolated by starch block electrophoresis (Fig. 1). The immunosorbed HDL separate into two zones of migration, alpha

and pre-beta mobility. Apo A-II migrates only in the alpha zone. No apo B was detected in the pre-beta zone, indicating that the apo A-I in this zone was not associated with very low density lipoproteins (VLDL). The isolated fractions retain their respective mobilities upon re-electrophoresis, implying that the fractions are distinct entities.

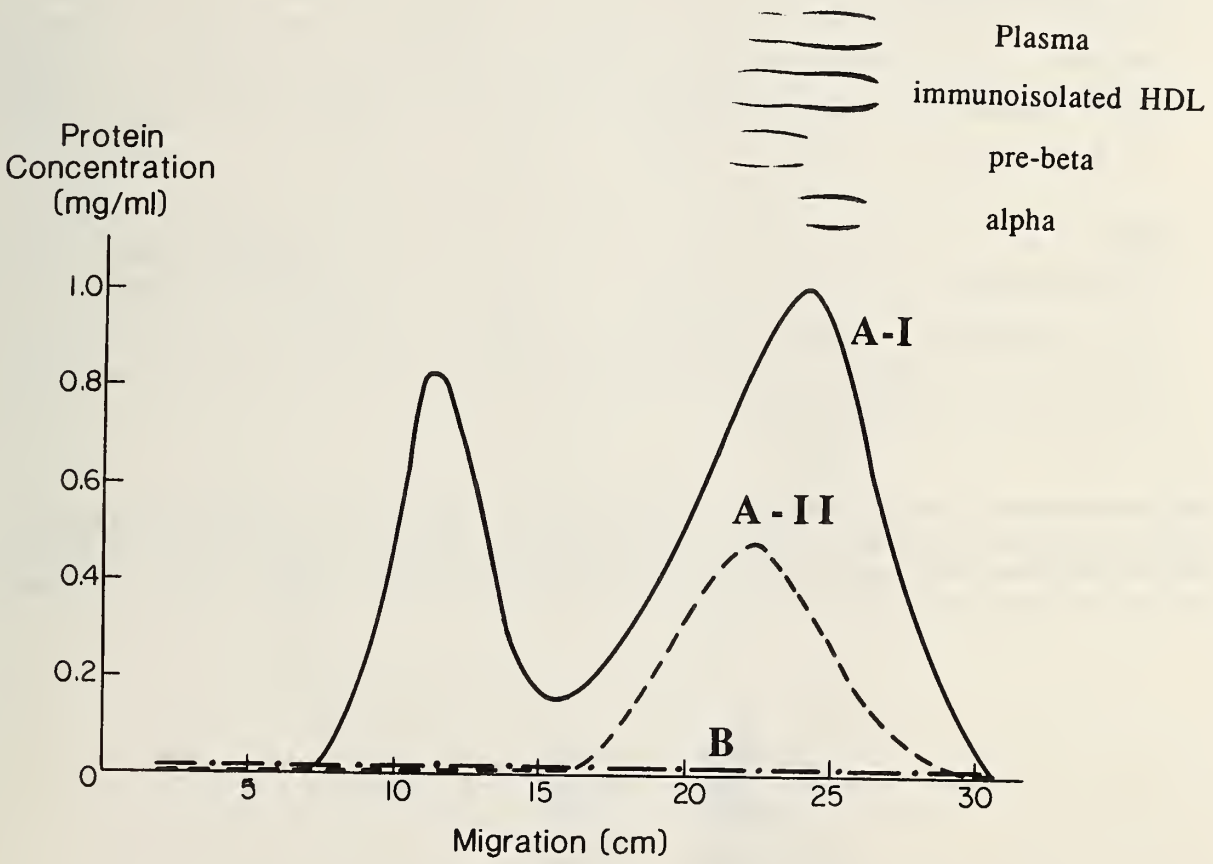

Figure 1. Starch block electrophoresis of immunosorbed HDL. After HDL were subjected to electrophoresis in a starch block matrix, the block was sliced into 1 cm fractions and analyzed for apo A-I (——), apo A-II (---), and apo B content. Inset: immunoelectrophoresis of plasma, HDL, and isolated pre-beta and alpha HDL against anti apo A-I-antisera, demonstrating the existence of pre-beta HDL in plasma and the retention of mobility after purification.

These pre-beta HDL are comprised of 90% protein. They also contain phospholipid and cholesterol, but no detectable triglyceride (Table I).

Table I. Percent chemical composition of the apo A-I-containing lipoproteins

	Protein	Phospholipid	Free cholesterol	Cholesterol ester	Triglyceride
Subject 1					
Alpha population	69.1	18.9	1.2	11.4	3.7
Prebeta population	93.0	6.0	.3	.7	ND*
Subject 2					
Alpha population	64.0	18.0	3.0	13.0	2.0
Prebeta population	93.0	4.8	0.1	2.1	ND
Subject 3					
Alpha population	65.0	18.0	1.0	12.0	4.0
Prebeta population	88.0	9.0	0.5	2.5	ND

*Not detected at a level of 25 ug/ml.

The predominant protein appears to be apo A-I, with some smaller molecular weight components (Figure 2). The particles appear to have a size of about 60,000 daltons when measured by size exclusion chromatography or by gradient gel electrophoresis.

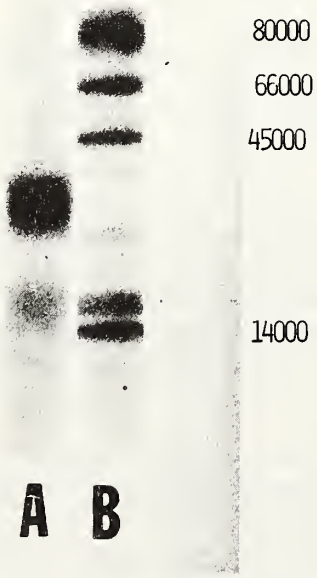

Figure 2. SDS gradient gel electrophoresis of the apoproteins of the pre-beta subpopulation were resolved on SDS gradient gels (A). Marker proteins are displayed for comparison (B).

While the pre-beta HDL have been visualized by several investigators (29-32), they have not previously been isolated cleanly. Other researchers have noted small HDL that contain only apo A-I (33-36) but have not identified these HDL as pre-beta HDL. In no case has this HDL subspecies been investigated further or has biological significance been determined.

Along with the compositional uniqueness of pre-beta HDL, their apo A-I has apparently assumed a conformation that is quite different from that of apo A-I in the bulk of HDL. When analyzed by circular dichroism, the protein in pre-beta HDL contained 52% helix compared to 62% helix of alpha mobility HDL (Fig. 3).

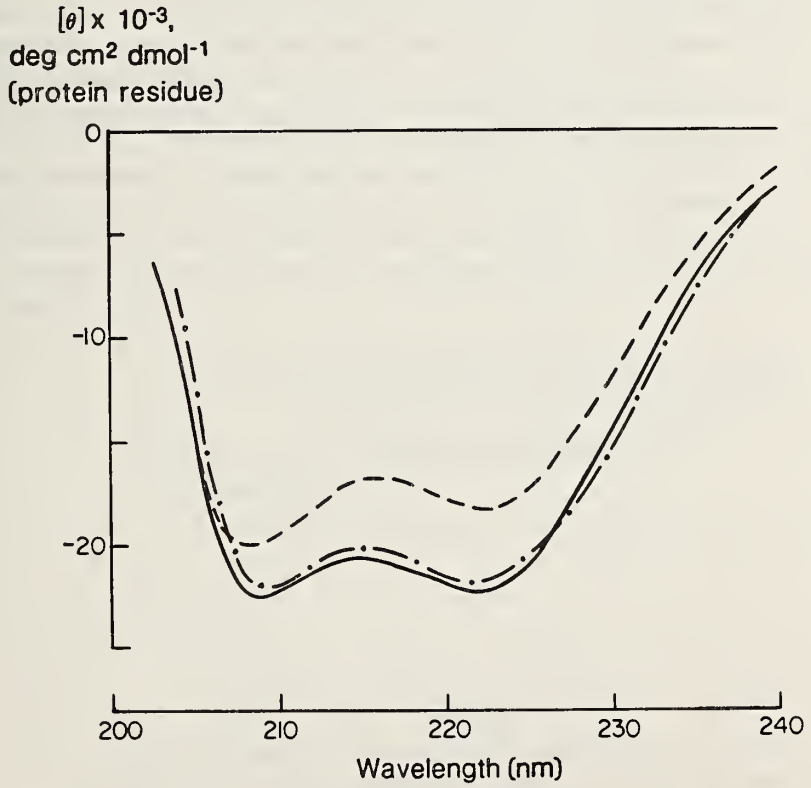

Figure 3. Circular dichroic measurements of immunosorbed HDL. The ellipticities of (A) ultracentrifugally isolated HDL (——), (B) alpha mobility subpopulation (-•-), and (C) pre-beta subpopulation (---) were measured from 240 to 200 nm. The helicity of each as determined from ellipticity at 222 nm was (A) 66.4%, (B) 64.3%, and (C) 52.0%.

Pre-beta HDL also react differently from alpha-migrating HDL with monoclonal antibodies against apo A-I. In liquid phase competiton assays pre-beta HDL were less potent inhibitors of the binding of ^{125}I-labeled HDL to selected monoclonal antibodies (Curtiss, Kane, and Kunitake, unpublished results), implying that some apo A-I epitopes are not exposed to the same extent in pre-beta HDL as they are in alpha HDL. In addition, we have found

that the apo A-I in pre-beta HDL is more sensitive to proteolytic degradation than the apo A-I in alpha-migrating HDL. In fact, the proteolytic fragments of apo A-I are the low molecular weight peptides found in pre-beta HDL (Figure 2). The implication is that the apo A-I in pre-beta HDL is in a different conformation from the apo A-I in alpha HDL and is more susceptible to proteolysis. This finding is of particular interest because recently, Gregg et al. (37) reported that proteolytically cleaved apo A-I is more rapidly removed from circulation than undegraded apo A-I, inferring that the amount of pre-beta HDL in the circulation may influence the rate of turnover of apo A-I. Such a possibility could explain the mechanism by which apo A-I is selectively degraded relative to the degradation of HDL-cholesterol in circulation observed by other investigators (38).

The structural differences between pre-beta and alpha-migrating HDL apparently have led to differences in their biological activities. We have found that pre-beta HDL and alpha HDL interact differently with bovine liver membranes (Table 2). The same difference is noted when human liver or fibroblast membranes are tested (unpublished results). The measured dissociation constant for pre-beta HDL binding to membranes was two orders of magnitude greater than that for alpha HDL, as determined by competition with labeled E-free HDL_3 for membrane binding, indicating that the epitopes responsible for the recognition of HDL membrane binding sites are masked or absent on pre-beta HDL, and that there probably is no physiologically relevant interaction between pre-beta HDL and tissue HDL binding sites.

Table 2. Dissociation constants of human HDL subfractions for the bovine hepatic HDL binding sites.

Lipoproteins	Kd (ug protein/ml)
HDL_3	3.3
HDL_2	1.7
Immunoisolated HDL	3.9
Pre-beta HDL	130.0

The dissociation constants were calculated from competition curves (28).

Of further interest is our recent finding that pre-beta HDL are converted to alpha-migrating HDL upon incubation at 37° (unpublished results). This conversion appears to be dependent upon lecithin-cholesterol acyltransferase (LCAT) activity because the addition of 1.4 mM DTNB or 50 mM menthol, inhibitors of LCAT, blocks the conversion. This finding, when combined with reports of a similar HDL subspecies found in LCAT-deficient patients (39) and the observation that HDL containing apo A-I only are necessary for cellular cholesterol efflux (40), points toward a role in the cholesterol removal process for pre-beta HDL.

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FACTORS AFFECTING THE FLUX OF CHOLESTEROL BETWEEN CELLS AND HIGH DENSITY LIPOPROTEIN *IN VITRO*

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Introduction

There is a well established negative correlation in humans between the incidence of atherosclerosis and the plasma level of high density lipoprotein (HDL) (for reviews see 1-3). This negative correlation probably arises from the participation of HDL in reverse cholesterol transport (4), the process by which excess cholesterol is tranported from the arterial intima and other peripheral sites to the liver for excretion. A central feature of reverse cholesterol transport appears to be the transfer of free cholesterol (FC) between cell plasma membranes and HDL (for reviews see 5-8). In tissue culture, the transfer of FC between HDL and cells is bidirectional and often close to an exchange of FC mass (9-13). The same sort of bidirectional movement must also occur *in vivo*. Thus, for HDL to promote cholesterol removal from peripheral cells, FC efflux from these cells to the lipoprotein must exceed influx. Likewise, if surface transfer is a mechanism by which HDL delivers FC to the liver, then influx from HDL to hepatic cells must exceed efflux. This aspect of reverse cholesterol transport depends critically on the balance of FC flux between cells and HDL.

The transfer of FC between cells and lipoproteins occurs without the expenditure of metabolic energy--that is, it appears to promote an equilibration of FC between HDL and the cell surface (for reviews see 14, 15). Therefore, the net movement of FC either to or from HDL requires the existence of a free-energy gradient for FC between HDL and the cell surface. Two types of occurrences might cause the generation of such gradients: 1. the relative depletion of FC in the prospective acceptor compartment, or 2. the relative depletion of phospholipid (the apparent solvent for FC in membranes and lipoproteins) in the prospective donor compartment. Several metabolic processes might result in such changes, as outlined in Table I.

Table I. Metabolic processes contributing to the net flux of cholesterol between cells and HDL.

Processes promoting net efflux from cells:

Depletion of FC from HDL, as mediatd by LCAT (10,16,17).
Increased cellular FC resulting from synthesis or lipoprotein uptake and degradation (18,19).
Cellular hydrolysis of stored cholesteryl esters (20-22).

Processes promoting net influx into cells:

Depletion of phospholipid from HDL, as mediated by hepatic lipase (13,23, 24).
Bile salt and steroid hormone synthesis.
Cellular ACAT activity.
Cellular phospholipid synthesis and expansion of surface membranes.

A major goal of our research is to understand the dependence of cholesterol flux on metabolically controlled compositional and structural features of HDL and cells. My presentation today reviews our studies on one aspect of this broad problem, the dependence of flux on the phospholipid content of HDL, as it is affected by hepatic lipase.

Methods

Our experimental methods are described in detail elsewhere (13,23,24). Our general approach is to study cholesterol transfer between HDL and cell monolayers. For the preparation of HDL, fresh human plasma is treated with 5 mM N-ethyl maleimide to inhibit lecithin:cholesterol acyltransferase (LCAT), and then total HDL is isolated in the density interval 1.063-1.21 g/ml by differential ultracentrifugation. Before use in experiments, the HDL is chromatographed on heparin-sepharose to remove traces of low density lipoprotein and apoprotein-E-containing particles. For most experiments, rat Fu5AH hepatoma cells are used. Several features make this cell particularly convenient for such studies: 1. FC flux between this cell and HDL is very rapid ($t_{1/2}$ for equilibration = 1-2 hours), allowing rapid completion of experiments in the absence of significant interference from ongoing cell growth or cholesterol synthesis and metabolism. 2. The Fu5AH cell does not endocytose HDL to any great extent (23), allowing the direct measurement of inward FC transfer from the lipoprotein. 3. The loss of cholesterol label from the cells (in the form of lipoproteins or bile salts) is

negligible in the absence of HDL or some other external acceptor, allowing the direct measurement of HDL-dependent FC efflux.

In several experiments, the major topic was the net transfer of FC mass from HDL to cells (23,24). These experiments involved relatively long-term incubations (8-24 hours) and standard chemical methods for the quantitation of cellular cholesterol and protein. In more recent experiments, we have used tracer methods to quantitate the bidirectional flux of FC between HDL and cells (13). For these studies, cells and HDL are pre-labeled with [^{14}C]cholesterol and [^{3}H]cholesterol, respectively, under conditions which almost completely prevent esterification of the tracers. Influx and efflux are observed simultaneously by monitoring the appearance of ^{3}H counts in cells and ^{14}C counts in medium. The results are analyzed by a computer program which fits the kinetics of redistribution of each of the tracers to a model for FC equilibration between two compartments (cells and medium). This analysis yields estimates of the rate constants for cholesterol influx and efflux (k_i and k_e) under a given set of conditions. The inward rate constant, k_i, is re-expressed in terms of clearance (Cl_i, units of ul time^{-1} [mg cell protein]$^{-1}$) in order to remove its dependence on medium volume and cell mass. The rate constants are used to calculate the influx and efflux of cholesterol mass (F_i and F_e) with the following relationships:

$$F_i = Cl_i \times [FC]_{med}, \text{ and}$$

$$F_e = k_e \times [FC]_{cells},$$

in which $[FC]_{med}$ and $[FC]_{cells}$ represent FC concentration in the medium and the cells, respectively, at zero incubation time. This experimental approach, although somewhat complex, offers some important advantages over previously described methods:

1. The measurements of influx and efflux are simultaneous and direct. The comparison of influx to efflux is perfectly controlled, and, at least at early incubation times, the measurement of the rates should not be affected by the cellular synthesis and metabolism of cholesterol.

2. Neither measurement depends on changes in the distribution of FC mass between cells and HDL. Long term incubations are unnecessary for the demonstration of net cholesterol flux.

3. The mathematical analysis is explicit for the existence of two interchanging FC pools. Thus, the method properly accounts for the back flux of cholesterol tracer from the acceptor compartment at incubation times beyond $t = 0$.

4. The method yields values for both the rate constants of cholesterol flux and the fluxes of cholesterol mass. This permits us to determine whether an effect on cholesterol flux arises from an altered FC pool size or an altered tendency (rate constant) for FC to move between compartments.

The kinetic methods were validated using our major cell model, the rat Fu5AH hepatoma cell. For purposes of this validation, FC tracer redistribution between total human HDL and Fu5AH cells was followed to complete equilibration. Equilibration required approximately 400 min of incubation (Fig. 1 of ref. 13). At equilibirium, both tracers redistributed identically, suggesting that all FC in both cells and HDL was available for transfer. When the data from the entire time course of this experiment were fitted by computer to the model for equilibration between two pools, the derived curves conformed to the data very well. This indicated that the fitting program worked reliably, and that the modeling in terms of just two kinetic pools was adequate for this system. Additionally, when data from only the intitial 90 min of the experiment were used for the kinetic analysis, the equilibration curves predicted the entire time course of the experiment within an uncertainty of 15%. This result suggested that we could limit experiments to a relatively short duration, and still obtain a reliable characterization of cholesterol tranfer between HDL and the hepatoma cells. This sort of approach seemed desirable, since it would allow us to study cholesterol flux with only minimal interference from cell growth, cholesterol synthesis, and cholesterol metabolism. In most subsequent experiments using the hepatoma cells, we have limited the kinetic analysis of data to time courses of 100 min or less.

Results

Depletion of HDL phospolipids mediated by hepatic lipase. Hepatic lipase is an externally oriented lipase of liver endothelium with specificity for the fatty acyl ester bond at the sn-1 position of neutral- and phospho-glycerides (for reviews see 25,26). Because of its external orientation, it is bathed in blood plasma and is in a position to influence the metabolism of plasma lipoproteins. When hepatic lipase is inhibited *in situ* in the rat by the infusion of specific antisera, numerous effects on plasma lipoprotein metabolism have been observed. Among these effects is that the content of phospholipid and free cholesterol in HDL is increased (27,28). Based on this observation, Jansen and Hulsmann (29) suggested that hepatic lipase may contribute to reverse cholesterol transport by depleting HDL of phospholipid and, thereby, indirectly promoting the partitioning of FC from HDL into hepatic cells. This hypothesis formed the basis for extensive studies in our laboratory on the effect of hepatic-lipase treatment of HDL on the transfer of cholesterol from the lipoprotein into cultured rat hepatoma cells.

Total human HDL was treated with rat hepatic lipase, post- incubated with bovine serum albumin to remove the products of lipolysis, and then reisolated by ultracentrifugation. A control HDL was treated identically, except that hepatic lipase was first inactivated by heating. The treatment with active hepatic lipase resulted in removal of as much as 28% of the HDL phospholipid, with little other apparent change in lipoprotein composition (Table II).

Table II. Effect of modification with hepatic lipase and bovine serum albumin on HDL composition. Values are the means ± 1 SEM for seven independent preparations of HDL. (Data calculated from Table 1 of ref. 23)

Compositional Parameter	Control HDL	Modified HDL
FC/protein (w/w)	Ø.Ø65 ± Ø.ØØ5	Ø.Ø67 ± Ø.ØØ6
total cholesterol/protein (w/w)	Ø.29 ± Ø.Ø2	Ø.31 ± Ø.Ø3
phospholipid/protein (w/w)	Ø.59 ± Ø.Ø1	Ø.51 ± Ø.Ø2
FC/phospholipid (m/m)	Ø.22 ± Ø.ØØ3	Ø.26 ± Ø.Ø1

The effects of control and lipase-modified HDL on cellular cholesterol content were compared. Incubation with the control HDL resulted in little change in cellular FC and a slow accumulation of cellular cholesteryl ester (Fig. 1 of ref. 23). These changes suggested a slow net inward flux of FC from the lipoprotein, which fueled the gradual accumulation of cholesteryl esters. This accumulation probably was augmented to a small extent by the synthesis of cholesterol and the endocytotic degradation of whole lipoprotein particles. Incubation with the lipase-modified HDL caused a dramatic accumulation of cellular cholesteryl ester, accompanied by a moderate increase in the level of cellular FC. The effects of the modification of HDL suggested that the net inward movement of FC was increased, and that the cells adjusted to this increase by accelerating the esterification of cholesterol. This adjustment minimized the accumulation of FC, but caused a large accumulation of cholesteryl ester.

Several questions were addressed in order to confirm this interpretation of the results:

1. Does the excess cellular cholesterol which accumulates in the presence of the modified HDL arise from the FC of the lipoprotein? This was confirmed using HDL which had been pre-labeled with [^{3}H]FC by celite exchange (Table 2 of ref. 23).

2. To what extent can HDL endocytosis and degradation account for the accumulation of cellular cholesterol? This question was addressed using HDL doubly labeled with [^{3}H]FC and ^{125}I-apoproteins. Lipase modification had no effect on the extent of apoprotein uptake and degradation, which, in any case, suggested that less than 3% of the FC delivery to the cells was by endocytotic mechanisms (Table 3 of ref. 23). Both the normal transfer of FC from HDL to the cells and the enhancement of transfer induced by lipase modification appear to be almost completely independent of lipoprotein uptake and degradation.

3. Does some of the cellular accumulation of cholesterol arise from the delivery of cholesteryl ester directly to the cells? The previously described results argued against significant delivery via particle uptake and degradation. Subsequent studies used a reconstituted HDL containing [^{3}H]cholesteryl oleoyl ether (a nonmetabolizable analog of cholesteryl ester) and [^{14}C]FC to address the question (24). With a control lipoprotein, the results suggested that in a

24-hour period the delivery of FC mass from the lipoprotein was three times greater than the delivery of esterified cholesterol mass. Hepatic lipase modification stimulated the delivery of the cholesteryl ester analog by 21% and the delivery of FC by 27%. In terms of cholesterol mass, these stimulations corresponded to an enhancement of FC delivery that was at least four times the enhancement of esterified cholesterol delivery. Thus, the results indicate that the direct transfer of cholesteryl ester from HDL may influence levels of cell cholesterol, but that the accumulation of cholesterol induced by the lipase modification of HDL is largely attributable to the enhanced delivery of FC.

4. Does the enhanced net tranfer of FC from HDL to the cells arise from the removal of the lipoprotein's phospholipid, or from some other undetected effect of hepatic lipase? The primary importance of phospholipid removal was confirmed by producing essentially the same effects with the specific phospholipase A_2 isolated from venom of Crotalus adamanteus (23,24).

In the most recent work on this problem, we have tried to account for the cellular acccumulation of cholesterol in terms of lipase-induced changes in the two unidirectional fluxes of FC at the cell surface (13). This approach employs cells and HDL which are pre-labeled, respectively, with [^{14}C]FC and [^{3}H]FC. Influx and efflux of FC are studied simultaneously by monitoring the transfer of ^{3}H counts to cells and of ^{14}C counts to medium. In a representative experiment from this work, HDL was labeled with [^{3}H]FC, and then modified by exposure to phospholipase A_2 (from venom of Crotalus adamanteus) in the presence of Ca^{2+}. The Ca^{2+} chelator EDTA was added to terminate the modification, and then bovine serum albumin was added to remove the products of lipolysis. A control (unmodified) HDL was treated identically, except that EDTA was present throughout the procedure. Both HDL preparations were reisolated by ultracentrifugation. Exposure to active lipase removed 50% of the lipoprotein's phospholipid, without affecting other compositional parameters (Table III). Comparisons of FC flux in the presence of control and modified HDL were made by diluting the two HDL preparations to equal protein concentrations. This resulted in equal concentrations of FC for a given comparison, but a lower concentration of phospholipid in the medium containing modified HDL. Media were applied to cells which had been pre-labeled with [^{14}C]FC, and the kinetics of [^{14}C]FC retention and [^{3}H]FC uptake were examined (Fig. 2 of ref. 13). These results indicated that phospholipid depletion of HDL reduced the initial rate of release of [^{14}C]FC, but had no effect on the initial rate of uptake of [^{3}H]FC. These results corresponded to a selective reduction in the rate constant for FC efflux (k_e). Data on FC flux obtained at several concentrations of control and modified HDL are summarized in Table IV. These results show that the selective reduction of FC efflux occurs over a wide range of HDL concentrations. Additionally, the results show that with HDL of normal composition (control), there is an approximate exchange of FC mass with the hepatoma cells ($F_i = F_e$), whereas with the phospholipid-depleted HDL, there is significant net influx of FC ($F_i > F_e$).

Table III. Effect of modification with phospholipase A_2 and bovine serum albumin on HDL composition. HDL were prepared and assayed as described in Johnson et al. (13). Values without uncertainties are the means of duplicate determinations. Values with uncertainties are the means ± 1 standard deviation of triplicate determinations.

Compositional Parameter	Control HDL	Modified HDL
FC/protein (w/w)	0.041	0.042
total cholesterol/protein (w/w)	0.26	0.26
phospholipid/protein (w/w)	0.37	0.19
FC/phospholipid (m/m)	0.22 ± 0.02	0.43 ± 0.08

Table IV. Effect of phospholipid depletion of HDL on the bidirectional flux of FC between HDL and Fu5AH rat hepatoma cells. HDL were as described in Table III. All values are the means of duplicate determinations and were calculated from data of Johnson et al. (13).

HDL concentration[a]	Control or Modified HDL	10^3 k_e[b]	Cl_i[c]	Initial FC flux[d]	
				efflux	influx
120	control	3.1	10.4	59	55
	modified	1.8	8.7	34	43
1200	control	12.6	5.1	237	253
	modified	9.5	4.9	178	240
2400	control	17.9	3.6	336	358
	modified	12.6	3.4	238	341
3500	control	22.0	3.1	414	457
	modified	16.4	2.8	308	414

[a] Units, ug protein/ml.

[b] Rate constant for FC efflux. Units, min^{-1}.

[c] Inward FC clearance. Units, ul min^{-1} (mg cell protein)$^{-1}$.

[d] Units, ng FC min^{-1} (mg cell protein)$^{-1}$.

The primary mechanism for net uptake of FC from phospholipid-depleted HDL appears to be the selective reduction of k_e, the rate constant for FC efflux. Because the rate constant for influx is unaffected, the reduction of k_e perturbs the equilibrium of FC between HDL and the cell surface towards net cellular uptake of FC. The fact that efflux is sensitive to the phospholipid content of HDL is consistent with the view that phospholipid is the solvent for FC in lipoproteins and membranes (30,31), and with work showing that dispersed extracellular phospholipid can independently promote FC removal from cells (32-35).

Discussion

It is now well established that the net transfer of FC between lipoproteins and cells is affected by the FC/phospholipid ratio in the lipoproteins. Low ratios result in net efflux, whereas high ratios result in net influx (9,13,23,24,36). Regarding cellular lipid composition, it has been reported that enrichment of cells with cholesterol can result in net efflux of FC to normal HDL (19,20,37). Presumably, the basis for net efflux in this situation is an elevation in the FC/phospholipid ratio of the plasma membrane. However, there appear to be no direct experimental comparisons between net FC flux of the surface of a eukaryotic cell and the analyzed FC/phospholipid ratio of its plasma membrane.

The mechanism for FC transfer between cells and HDL is not entirely resolved. The movement of FC between cell surfaces and artificial acceptors (phospholipid vesicles and phospholipid-taurocholate mixed micelles) is by the diffusion of cholesterol molecules through the intervening aqueous space (32,38). The efflux of cellular FC promoted by such acceptors is similar to the efflux promoted by native HDL (13) and by recombinants of phospholipid and HDL apoproteins (35). Thus, it seems likely that a large component of FC transfer between cells and HDL occurs by the aqueous diffusion of the lipid. HDL binds specifically and reversibly to several types of mammalian cells with K_{dis} in the range of 2-90 ug protein/ml (39-51). Brinton et al. (40) recently reported that when specific HDL binding was prevented, there was a significant reduction in the efflux of FC from fibroblasts to low concentrations of the HDL (3-30 ug protein/ml). This result suggests that FC transfer between cells and low concentrations of HDL may be mediated by the specific binding of the lipoprotein to cells. Such mediation could occur by two possible mechanisms: 1. The binding could allow a protein-mediated specific exchange of FC between HDL and the plasma membrane. 2. The binding could promote the aqueous diffusion of FC between the lipoprotein and the cell surface by producing a high local concentration of HDL in the immediate vicinity of the plasma membrane.

Hypotheses on the functional significance of FC transfer between cells and lipoproteins now extend in two general directions. Firstly, FC transfer between cells and HDL is established as an essential part of the pathway for reverse cholesterol transport. Secondly, FC transfer processes may be involved in certain pathological processes. According to the recent proposal by Fielding (52), an important feature of noninsulin-dependent diabetes and some forms of hyperlipidemia is an elevation in the concentration of FC within plasma lipoproteins. Fielding suggests that the high incidence of atherosclerosis in these

groups may be caused by the uncontrolled net transfer of FC from these lipoproteins into arterial intimal cells. This hypothesis is consistent with work by Kuksis et al. (53), which suggests a positive correlation in humans between the FC/phospholipid ratio of blood plasma and the incidence of ischemic vascular disease.

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DR. DECKELBAUM: Dr. Barter, we've modified HDL2 and HDL3s with Intralipid and we found that there were two mechanisms involved in making HDL grow. One was triglyceride enrichment and the other in vitro was phospholipid enrichment, especially if you use the commercial preparation which hasn't been washed before the incubation. When we infuse Intralipid in vivo we find that the enlargement of HDL3 and HDL2 is due solely to phospholipid enrichment without necessarily increasing the triglyceride. So, we believe that during the infusion in the in vivo situation we would agree with you that hepatic lipase is very active. Actually, Lawrence Carlson presented data a week and a half ago which was interesting in relationship to his hepatic lipase deficiency patients who had a moderate triglyceridemia of about 300 milligrams percent. These patients had a lot of triglyceride in their HDL and all their HDL was large HDL2. They were in fact very cholesteryl ester depleted, almost no cholesteryl esters in their HDLs. So, these hepatic lipase deficiencies I guess were the human model for the rabbit. In terms of the intralipid enrichment, you can have both mechanisms going on.

DR. BARTER: I would agree with that, but we find that the triglyceride enrichment is real and we get very similar results in terms of the particle size in vitro when we use VLDL. We get more phospholipid enrichment with the Intralipid, but we still get the same general result with the LDL.

DR. NESTEL: Dr. Barter, would you want to consider the possibility that low or almost absent concentrations of very small particles are not due to their being rapidly replenished with cholesteryl ester in their core, but rather that being relatively triglyceride-rich and, as you pointed out, excellent substrates for hepatic lipase that they may be cleared much more rapidly from circulation?

DR. BARTER: That's possible, but the small particles are made by two different mechanisms and in the hypertriglyceridemic subjects you may well be right and that would fit in with some of your studies of the A1 turnover in hypertriglyceridemia which is an accelerated turnover. The particles which I think are involved in the LCAT reaction are those generated by the conversion protein and they have virtually no triglyceride in them at all.

DR. SCANU: We became aware of the work by Kunitake some time ago and we have already made a similar observation which shows that by independent methodology we can also demonstrate the pre-beta HDL, particularly in hypertriglyceridemic subjects. We were familiar with the single spin technology that is a density from 103 to 115 in this case. In normal subjects, we collect fractions from the single spin and then run the HDL3 on agarose gel electropheresis. In a normal lipidemic subject, you can see only alpha-1 migrating HDL with one percent agarose a simple technology, no immunoaffinity, or anything. But if you now take hypertriglyceridemic subjects, you have values of cholesterol and triglycerides, IDL values. By taking the fraction here you can see a pre-beta migrating band. It is from the HDL. Here is another subject here, the cholesterol here. Now, what we need to ask ourselves is whether if we take all the HDL line and can see them as a function of the hypertriglyceridemia, you can see that the HDL contains the pre-beta band. We have immunoblotted all of this and we show that only the pre-beta contains A-1 whereas the alpha-1 migrating contains ApoA1 and A2. So, we confirm by independent methodology the work by Kunitake.

DR. KUNITAKE: I'd like to comment about this in relationship to Dr. Barter's very nice presentation. In hypertriglyceridemics and incubation with Intralipid, Dr. Barter may be seeing a derivitized form of pre-beta HDL that has become loaded with triglyceride and that is why you can see it with a lipid stain by Dr. Scanu's technique, but you cannot see it with a lipid stain under normal conditions.

DR. SCHONFELD: I reported some data in the journal "Lipids", the proceedings of the American Oil Chemists Society meeting in St. Louis sometime in the 1970's. I filtered plasmas on agarose columns and assayed ApoAl levels across the columns, trying to find what in those days I called "free ApoAl", to see if there was any free ApoAl in plasma because there was a question at that time whether all Al was associated with HDL or if it was associated with some other fraction. I found that in normal plasmas there was a very small percentage, maybe two or three percent that came out in an area that had an apparent molecular weight of about 50,000. In other words, it was around a little bit lower than albumin. But, as the hypertriglyceridemic plasma was filtered more and more free ApoAl came out. There was a graph in that paper which had log triglyceride concentration on the ordinate and "free Al" on the abscissa and there was a pretty good relationship between the apparent amount of free Al as a percentage of total Al in the triglyceride level. That's why I'm glad to see that this is all kind of hanging together.

DR. SCANU: You said with the free Al?

DR. SCHONFELD: Well, I don't know what it was. I didn't characterize it. It was just a much smaller--now, I never found any A2 in that area by immunoassay across those columns.

DR. KUNITAKE: I'm very aware of that work. I even cited it. I didn't go into that matter further because Christopher Fielding and Brian Ishita in a publication that will be published shortly have investigated the level of pre-beta and triglyceride levels in plasma and they indeed find that correlation does exist.

DR. SCHONFELD: Good. So, do you think this is the precursor particle?

DR. BARTER: We've done some experiments which we haven't published with plasma from hypertriglyceridemic subjects from which we removed the VLDL and then incubated, just the whole system which is LCAT active, and then characterized the HDL particles. The big particles that occur have got more Al on them then would have been predicted from the original size which means either there's been a fusion, which is what I was thinking, or these pre-beta particles are providing essentially dimers of Al to a growing HDL particle and it would be absolutely consistent.

DR. KUNITAKE: I agree.

DR. SCHONFELD: Where would these particles migrate on these gradient gels? Do you have any idea?

DR. KUNITAKE: The pre-beta HDL migrates around 60,000 molecular weight. If you looked again at the gradient gels of Dr. Cheung and of Dr. Segrest, you see that they have a small band in that region. They didn't talk about it.

DR. SCHONFELD: With respect to the immunology, that was the other thing that we noticed. In order to see ApoAl in whole HDL, we had to delipidate or perturb the structure some how. But the "free Al" was virtually all visible. This is by polyclonal radioimmunoassay. So, the polyclonals must be seeing an altogether

different epitope from what you are seeing. Of course, we use labeled A1 also in those assays. So, that epitope that was being probed there was much more seen than intact HDL.

DR. PACKARD: This may help explain something that we say that Simon Rouck just published that normally, if you look at the metabolism of A1 and A2, the A1 goes slightly faster than the A2. But in severe hypertriglycerdemia they saw that the metabolism of the A2 was much faster than the A1, about twice as fast as A1. So, it is possible that this A1 particle is slowly metabolized and it has a distinct metabolic rate from the A1/A2 particles in the plasma of these subjects.

DR. KUNITAKE: This may also help to explain the results of VanTol in which he finds there is a dissociation in the kidney between the metabolism of ApoA1 and HDL cholesterol.

DR. ALBERS: On that issue, could you speculate on the physiological origin of this particle? Would you comment on the relative quantitative distribution in different biological states that may give us a clue.

DR. KUNITAKE As far as solid scientific foundation, I have almost none. So, I'm perfectly happy to speculate, but don't hold me to it. The pre-beta HDL does not contain any pro-form of ApoA1. That's the first thing we looked for. We thought perhaps may be this is a pro-form since it is found in lymph and it is also found in HEP-G2 cell incubation medium. However, those two findings are dangerous because discoidal particles containing ApoA1 also migrate in that region. As far as any clinical subjects which may shed light on this particle, we have found that both in A-beta lipoproteinemics and in patients who have had their small bowels surgically removed there is no pre-beta HDL in the plasma of these patients. Also, we have the patient that produces only B-48 and not B-100 that Mary Malloy and John Kane have described so beautifully. This patient does have a smaller amount of pre-beta HDL. So, it is possible that there is intestinal input of this particle.

DR. INNERARITY: It is important to note on this very interesting data that in that gradient gel run that Dr. Kunitake presented where it was only delipidated A1, although you have evidence of oligomeric forms there, you don't have the dimer migrating in that region. So, in the particle that contains 93 percent protein plus seven percent lipid it would seem that the lipid component is a strong organizing principle with respect to the properties that are exhibited, i.e. the pre-beta mobility and the intriguing conversion properties that it has. So, albeit it is 93 percent protein, it migrates in the region that is dimeric. You don't find that in ApoA1 by itself.

DR. KUNITAKE: That's a very exciting structural finding. We also came to that conclusion but since this is supposed to be a metabolic talk, I didn't really go into that too much.

SPEAKER: Dr. Kunitake, you mentioned that the amino terminus was observed with both the degradation product that you saw with the plasma degradation, either with the plasma or any other protease that these may have tried. Does it appear that this is a discrete cleavage?

DR. KUNITAKE: In the pre-beta HDL, that is the material we isolate from circulation, this appears to be discrete. In the first stages of plasma degradation, this appears to be discrete. However, we have found that A1 and pre-beta is completely degraded

by plasma and if you incubate long enough--that's why the incubations were only three minutes and six minutes. We have also found with other proteases that pre-beta HDL is much more susceptible to proteolytic degradation.

DR. HAVEL: I wanted to tell Dr. Schonfeld that pre-beta HDL was first observed by Fredrickson in the '60s.

DR. ORAM: Dr. Johnson , first of all, it is likely that you're not looking at receptor-mediated processes with your cholesterol load itself for two reasons. In our studies, using HEP-G2 cells we found that the expression of the binding protein was very, very low and that cholesterol loading does not actually affect its activity. So, it is likely that in hepatoma cell lines very low protein activity is expressed and it's not upregulated by the levels of cholesterol achieved. Even if cultured fibroblasts were used to get upregulation of HDL binding, you must have very extensive loading and be very careful that you're in that range in which the HDL receptor activity is being upregulated. So, you have to be careful not to draw conclusions that this indicates that the receptor processes are not involved in the efflux of cholesterol from cholesterol-loaded cells, unless you do parallel binding studies to make sure.

DR. JOHNSON: We haven't done the binding studies yet with hepatoma cells. We have done some experiments with fibroblasts, but we are really not ready to present those results. They are more preliminary right now. It appears from those results though that the same trend is apparent as with the hepatoma cells.

Dr. Havel: I'd like to make a few comments regarding liver in which there might be some clinical models and relate our thinking back to some other systems where there is flux of lipid between liver and plasma. One of these is the FA albumin system which has been extensively studied and there has been a lot of research on the possibility that there is an albumin receptor involved that is responsible for uptake of FFA. That now has been extensively studied by Weisiger and his colleagues at UCSF after earlier work by him and by Ochner and others that proposed an albumin receptor because of saturability and certain other properties of the uptake system. Those are papers published in the last year or two. They concluded there is no albumin receptor in the liver.

The second process I'd like to refer to in a different system in the uptake of thyroxin by liver. This is work that Mendel and Weisiger recently published using autoradiography. They found that the site of uptake of thyroxin in the liver in the absence of thyroxin binding globulin (TBG) is almost entirely into the perforated sinusoid and no uptake is observed toward the center at all. But, if they bind thyroxin to TBG, then a very nice equalization of uptake is observed all along the sinusoid and a similar thing seemed to apply to the FFA uptake. Therefore, it is possible that another function of HDL in the flux of cholesterol between cells and plasma, not just liver, is to equalize the chemical potential in the various parts of the body such that equilibrium between cells and plasma is maintained. So, an additional function of HDL might be thought of as an equilibrium rather than promoting flux solely, although obviously it would depend upon the chemical potential. If there is this equilibrium, that flux one way or the other won't occur. I just thought I'd put that into the equation as a new way of thinking of these functions of binding proteins and the way HDL may be a binding protein for free cholesterol.

DR. JOHNSON: I agree with you. What you say is very reasonable and that there could be just very subtle differences in the chemical potential between cells and HDL. So you have a small net component of flux at any given time and that could be sufficient for mediating the movement of cholesterol between the periphery and the liver.

DR. INNERARITY: From what I recall in the article in Methods of Enzymology is that you all have looked at a number of different cell lines for efflux. Have you looked at endothelial cells where the largest amount of HDL receptor is found? If you have, did you see the same thing or something different?

DR. JOHNSON: No, we haven't looked at endothelium. I'm not sure if efflux experiments were done with endothelium-- were they with endothelium?

SPEAKER: It was a bovine endothelial cell line, but we just gave half-times for efflux. They were relatively long, but there were no bidirectional studies done with endothelium.

DR. NESTEL: Dr. Johnson, could you clarify the answer that you gave to Dr. Oram? You said that you had not done formal binding studies, but in an earlier slide you said that the flux of cholesterol between HDL and the cells was independent of any uptake of HDL protein that you'd labeled. Have you compared that with normal rat liver cells?

DR. JOHNSON: Well, we really don't know in our experiments where we have enriched cells with cholesterol whether we have affected HDL binding. That is the important thing we wanted to look at there. So, the earlier experiments where HDL uptake and degradation were looked at, those were just cells with a normal level of cholesterol in which the HDL was the variable rather than the cells. Regarding whether binding is promoting cholesterol flux, we want to look for some correlation between the rate constants for cholesterol flux and the extent of HDL binding and, since we haven't done the measurements for HDL binding as a function of cholesterol enrichment we expect with cholesterol enrichment for binding to be increased and therefore, if this binding is promoting cholesterol flux then the rate constants for flux would be increased. So, if we don't see that correlation between the extent of binding and the rate constants for flux, we would conclude then that binding is probably not promoting cholesterol flux.

DR. DECKELBAUM: Most of us sample plasma in the laboratory from a set of donors by taking a 12-hour fasting sample. However, most of the day people are actually walking around in a post-prandial state. How would you reconcile your findings? People with upregulated lipase systems are supposed to, in fact, be in better health and those same people, in fact, after a meal transfer more phospholipids into their HDL. So, in fact, in vivo you are going to be operating most of the day by two mechanisms. You are going to have your active lipase which will keep cholesterol in the cells and at the same time you are increasing the phospholipid content of your HDL if you are healthy and that's going to be a factor.

DR. JOHNSON: Well, the idea with the lipase is that its effect on cholesterol flux is going to be localized to the tissues where it resides. So, the idea was that hepatic lipase would modify HDL within the liver sinusoids and then a rapid partitioning of cholesterol from the HDL and the liver would occur. So not only phospholipid-depleted HDL would leave the liver so you wouldn't have a general effect of net inward movement of cholesterol onto all cells, all tissues in the body.

DR. RUDEL: Do your FU5AH cells or some other hepatic-derived cell have hepatic lipase that you could use to test your hypothesis directly with a lipase that's a component of the cell? Is that a possibility?

DR. JOHNSON: I don't know offhand. I don't think the hepatoma cells express a lot of hepatic lipase, if any, but I don't know the answer to that.

SPEAKER: There is a paper by Pierson saying that there are hepatic cell lines that secrete hepatic lipase.

DR. JOHNSON: You'd also want a cell where it was bound to the surface if you are going to test the hypothesis completely.

DR. ORAM: Have you looked at nitrated HDL and its effects?

DR. JOHNSON: Yes. What we've seen there is that in the low concentrations of HDL the nitration does not seem to have a big effect on cholesterol flux for us. So, those results are going to be different from yours. At higher concentrations of HDL, we do see a significant effect which is interesting; way out of the range where you'd expect binding to be important for cholesterol flux. That suggests that it is more perturbation of the structure of the particle that is important rather than an effect on binding.

SESSION X. Discussion of Lipoprotein Heterogeneity

Chair: Sandra Gianturco

SESSION X. DISCUSSION OF LIPOPROTEIN HETEROGENEITY

DR. GIANTURCO: First of all, I'd like to thank once again Ken Lippel for all of the hard work that he did in organizing this workshop and bringing it to fruition. I'd also like to thank the organizing committee and finally the speakers. We've got a lot of new information and a review of all of the old methods and the new methods. One of the purposes of this workshop is to recommend areas for future research. Therefore, I'm asking each of the chairmen to summarize or discuss what he thinks is important about the meeting or his own session and then we will hear from the audience on what you would like to see done in the future.

DR. GIANTURCO: I would like to say a few things about the opening session, session I, and couch some of this in terms of what is the atherogenic lipoprotein. Probably many of us in the opening session, believe it is going to be an ApoB-containing lipoprotein and very possibly Ron Krauss's LDL. Is there a particular atherogenic lipoprotein or is there a whole family? This topic will be open to much future research using the old immunoaffinity purification methods which have now gained a lot of new implications with the development of monoclonal antibodies. I would like to point out also that we don't know very much about the post-prandial lipoproteins and Richard Deckelbaum would like to say a few words about these.

DR. DECKELBAUM: (slide) That's a normal HDL zonal absolute pattern, HDL-2 and HDL-3. Now, we've heard for example today that you can perturb this normal system in chronic states like hypertriglyceridemia.

The first figure of how hypertriglyceridemia makes small HDL look like this came from an NHLBI workshop in which Dr. Joseph Patch presented that figure and it was published. That is the first figure where hypertriglyceridemia makes small HDL. We've published similar results. We've also shown another situation with no triglyceridemia in abeta in which very big HDL are at this end. You have an abeta something like this, called HDL-2. Dr. Joseph Patch also showed that you can perturb this system in people who were deficient at getting rid of their meals who also build up their HDL-2 in preference to their HDL-3 and there is a shift of both populations. At triglyceride levels of a thousand, an HDL population is seen that also moves up way ahead and very sharp peaks are moving the HDL population. So we observed subpopulations moving in different ways in response to LCAT, in response to triglyceride, and something that we didn't really talk about very much today is the response to phospholipids moving.

Another model, in addition to human illnesses and human genetic diseases, is to understand what is happening in normal plasma processes. What we are doing every day when we're eating. A given subject has a lot of perturbations of his lipoprotein system and lipoprotein heterogeneity. I think that this should also be followed.

DR. FISHER: I'd like to show a slide. This is a very typical sort of centrifugational run of serum total lipoproteins. You can see a little VLDL off on the left and on the right the HDL just beginning to appear and in the middle the LDL. LDL can certainly present itself, as we all know, in a variety of different states. Now, the reason I show this slide addresses the question that Dr. Chait just posed and that is to try to sort out some nomenclature. Last night, Dr. Krauss and I came to an agreement which will permit us to talk about different dispersions of LDL. We all recognize that LDL consists of a continuum of particles of different sizes, densities, but that if you look at the normal individual the majority of LDL exists within a very narrow density range. I had referred to this as monodispersed LDL; Dr. Krauss did not like that term at all and I agree with his objections. There is a polydispersion of lipoprotein particles throughout the spectrum. So, both of us agreed to suggest that we refer to this dispersion using the Latin term pauci to refer to small or few species and that we refer to such LDL as pauci-dispersed LDL. When we end up with a distribution of LDL like this where you have multiple peaks or even in this case where there are obviously several different components, we refer to as multidispersed LDL.

DR. SCANU: It is a semantic question.

DR. FISHER: It is a purely semantic question. At any rate, Dr. Krauss and I felt that we were communicating now and we wanted to just pose that to the group. One of the things that has been very exciting in our laboratory over the years has been the fact that the ApoB-containing lipoproteins are processed and Richard Deckelbaum and others have presented this so nicely. But perhaps there is another story here that needs to be emphasized. Let us look at just a little data regarding patients who have this pauci-dispersed distribution of LDL molecules. If you isolate LDL from individuals with pauci-dispersed LDL on repeated occasions and look at the molecular weight of this lipoprotein, the striking things are two-fold: there is a very wide range in the molecular weight of the LDL. It goes from around two million to four million daltons. The other very striking finding is that in a given individual it is almost constant on repeated occasions. What is there that permits the body to so tightly regulate the metabolism, the processing of LDL, that you get such tight reproducibility on repeated occasions? That has been an enigma which I don't think we ever really addressed. If you look at the dispersion of LDL in a variety of human subjects--here we have 77 subjects--this is the distribution of LDL molecular weights in these individuals. These were Caucasians here and a group of 44 blacks, again this wide distribution of pauci-dispersed molecular size in the subjects. If you take three individuals who had pauci-dispersed LDL and subjected them to a dietary perturbation looking at their LDL over 24 hours on five different occasions, fasting and after meals, on a variety of different cholesterol contents, the molecular weight of a given individual's LDL is almost invariant within the limits of the experimental methodology. If you take three different individuals and put them on a high cholesterol diet with a high level of saturated fat for one week and then for a second week on a low cholesterol diet with a lot of fat, on finds once again that the change in the molecular weight of LDL is really very little. Following through on Alan Sniderman's observations, if you sample LDL across the splanchnic bed from the aorta to the hepatic vein, and look at the molecular weight of LDL, the change of LDL as it transverses the liver, one sees very little change in a group of individuals with pauci-disperse LDL. How is it the body manages to carry out all of this processing that we've heard about and yet ends up with discrete species of LDL which are maintained so discretely? I'd like to pose that as one of the concerns that we have not yet addressed.

DR. HAVEL: This is a very vital field, looking at LDL structure and metabolism from the standpoint of heterogeneity of particles and I think it is a healthy field. The question of nomenclature may be handled better at the level of a few people sitting down and talking normally. We might make the proposal, other than the workshop, that there is no obvious need that I can see for crash programs in one aspect of heterogeneity or the other. I'd like to hear other people's ideas about that, but I was persuaded that the existing mechanisms appear to be fostering wittingly or unwittingly just because of the stage of technology and concepts, appear to be promoting a very healthy movement of the field in what I feel is the right direction.

SESSION II SUMMARY AND DISCUSSION

DR. NESTEL: In session II there were three papers that dealt with the uptake of triglyceride lipoproteins by macrophages, both VLDL of endogenous and exogenous origin. Dr. Gianturco and Dr. Krul spoke about those lipoproteins which were derived from dietary particles that I reviewed and to some extent the common interest was the possibility that this might be the mechanism in atherogenesis, although that was raised only during discussion. Namely, that these lipid-laden foam cells which initially are rich in triglyceride do also become enriched in cholesterol and during intracellular catabolism of triglyceride we may be left with cholesterol-rich particles.

Dr. Gianturco reviewed mainly the ligand/receptor interaction and her extensive work in which the larger particles were taken up through ApoE as ligand and with further delipidation through the ApoB ligands but she did not believe that the LDL receptor need be the site of binding because her thrombin-treated hypertriglyceridemic VLDL which still don't bind to the LDL receptor did, nevertheless, bind with high affinity to the macrophage.

Dr. Krul pointed out that this thrombin-accessible ApoE is not necessary for binding to the receptor, but it affects the binding of other ligands presumably from the ApoB species and is therefore significant. Dr. Krul supported some of Dr. Gianturco's work by showing with specific monoclonal antibodies that blocking ApoE prevented binding of large VLDL and reduced binding of smaller VLDL. Dr. Krul also discussed the heterogeneity of the ApoE epitope interactions with VLDL. She made several observations which were of considerable relevance. After all, if different epitopes of ApoE may be accessible to receptors to a different extent in different hypertriglyceridemic patients or even in the same patients studied on two different occasions, then clearly we would be in trouble. Also, the work that she described with the anti-ApoE immunoaffinity columns also might be important for those of us that want to use that technology for VLDL turnover studies because she pointed out that not all ApoE and VLDL is accessible to retention on the columns and this may of course also be important in vivo. Possibly part of this results from the amount of ApoC that remains associated with the VLDL. However, at least those VLDL that were capable of being retained by the column are also bound to the LDL receptor and I think that is an area that needs to be exploited and explored carefully by the in vivo metabolic people.

I presented some data on TRL metabolism from type five hyperlipoproteinemic patients and made the suggestion that the chylomicron remnants from these patients are in some ways resistant to lipolysis to the extent that the ligand that is responsible for the removal of those particles does not become accessible to the chylomicron receptor in the liver and since the same particles interacted as avidly as beta VLDL with macrophages, the possibility exists that this was possibly an alternate way of clearing those chylomicron remnants in those conditions and raise the possibility that it may be a factor in the generation of atherosclerosis.

SESSION III SUMMARY AND DISCUSSION

DR. NESTEL: In Session III, Dr. Weisgraber discussed HDL with ApoE and showed that, although it was present in low concentrations in man, it shared some of the size heterogeneity with the same particle seen in animals and pointed out that its' presence in plasma was inversely related to cholesteryl ester transfer activity and that may have a function not only in reverse cholesterol transport to the liver, but in redistributing cholesterol from cholesterol-enriched cells to those cells that may require cholesterol. He therefore raised the question whether this was a highly desirable type of particle or whether it was potentially an undesirable or atherogenic particle.

Tom Innerarity reviewed the evidence against the putative beta VLDL receptor, pointing out that failure to detect the binding of beta-VLDL to the B receptor was due to the fact this may be an unusual receptor, that 100-fold excess LDL may be required to displace beta-VLDL and finally gave evidence from anti-LDL receptor antibody studies and LDL receptor quantification to show that beta-VLDL binding is clearly related to that particular receptor.

Dr. Scanu then reviewed the physical and chemical properties of Lp(a) and very nicely highlighted the heterogeneity of Lp(a) within an individual and between individuals. He pointed out how little was known of its physiology, and stressed how important this knowledge might be in view of the strong correlation between Lp(a) concentration and atherosclerosis. He also emphasized that certain dietary interventions and cholestyramine treatment which had profound affects on ApoB concentrations failed to influence Lp(a) concentrations.

So, the sort of questions we can ask in these various papers were briefly alluded to. That is, do TRL in macrophages eventually lead to foam cell formation which compares in significance with foam cell formation that is inducible with beta-VLDL or chemically-modified LDL? What is the nature then of the macrophage response to this in terms of its subsequent potential to release powerful peptides that may be damaging to the endothelium? What are the implications of the heterogeneity in ApoE epitopes on hypertriglyceridemic VLDL to the metabolic channeling of those VLDL? Do we have enough information whether, in man, HDL with E serves an essential scavenger function for excess cholesterol transport? Does it become damaging? Do beta-VLDL particles in man, other than in type three patients, ever reach a concentration that is potentially damaging and do the small cholesteryl ester ApoE-enriched VLDL observed in people eating excess cholesterol resemble the metabolism of beta-VLDL? And finally Dr. Scanu made a very good plea for increased research into Lp(a) in view of its association with atherosclerosis.

SESSION IV SUMMARY AND DISCUSSION

DR. GRUNDY: Session IV was on the in vivo metabolism of ApoB-containing lipoproteins and was concerned mainly with the kinetics of VLDL and to a lesser extent with LDL. In my view kinetic modeling of VLDL is a very complex subject, to say the least. Unfortunately, we have not resolved all of the issues of how to put together the various input pathways and clearance pathways for VLDL that will enable us to get a totally integrated picture of VLDL metabolism which might allow us, for example, to estimate total production rates of ApoB which is an important question.

One of the facts that emerged was that large VLDL appear to be removed rather rapidly from the circulation with less conversion to LDL or a small VLDL preferentially converted to LDL. Clearly ApoE plays a role in modulating the catabolism and directing the fate of VLDL. It's apparent that the LDL or B/E receptor affects the clearance rate of VLDL remnants, but, as pointed out by some, the ApoE or chylomicron remnant receptor also may play a role which adds to the complexity of the question. One of the issues is whether we can actually measure production rates of VLDL ApoB accurately. This is an important question because, as discussed subsequently, the issue of hyperApoB or familial combined hyperlipidemia appears to be related to an over-production of VLDL ApoB. However, if we can't accurately measure the VLDL ApoB production, then we can't be absolutely certain about that and it is possible that there could be some metabolic channeling of the VLDL such that in this condition there are just more small VLDL made that make it look like there is an over-production of VLDL ApoB when in fact it is conceivable that the liver is not synthesizing more of ApoB itself and we certainly don't have any means right now to estimate in humans the amount of ApoB synthesized by the liver. That's just one example of where our uncertainty about correct models leads to uncertainty of interpretation in a very important area of hyperlipidemia. Obviously a lot more work needs to be done in that area. Dr. Ginsberg pointed out some of the difficulties in the methodological aspects of isolating VLDL. Dr. Packard did the same. It is clear that there has to be a tremendous amount more research done in this area to understand the pathways of VLDL production and metabolism.

SESSION V SUMMARY AND DISCUSSION

DR. FISHER: Compartmental modeling was the subject of session V. Dr. Zech presented a series of concepts regarding compartmental modeling which were very important for us. The importance of compartmental modeling in the representation of experimental data so that it may be presented in a fashion that can be understood in physiologic terms is becoming increasingly evident to all of us and it is tremendously important to recognize the power of this tool which we have available. Developing a model to constitute an analog of a physiologic system permits one to analyze data in physiologic terms, to generate hypotheses which can then be tested again in the laboratory. This contribution of compartmental modeling, as well as the hazards involved in so doing, were stressed and need to be stressed.

The talks by Dr. Gregg and Dr. Witztum, in one case looking at the metabolism of the ApoE isoforms and the other a very intersting system in which cholestyramine has been used as a perturbant of apoprotein B metabolism were both exciting presentations and very nicely focused the fact that lipoproteins differ strikingly in their metabolism, depending upon the composition of the particles, and that the metabolism of these lipoproteins may be readily perturbed by both specific pharmacologic and dietary alterations. There is obviously a great deal to be learned in the physiology as well as the pathology of lipoprotein metabolism by studying individual subjects under a variety of different physiologic and pharmacologic states. Each subject is in itself an experiment that needs to be analyzed and understood as one moves from one sort of perturbation to another.

A corollary to this is that there is a hazard that we need to recognize in terms of trying to group subjects in terms of clinical classifications when in fact there are so many metabolic differences among subjects within a given clinical classification. Perhaps we are moving into an era where it is going to be very important to examine individual subjects for what we can learn from them.

SESSION VI SUMMARY AND DISCUSSION

DR. GRUNDY: Session VI, was on dietary effects of heterogeneity of ApoB-containing lipoproteins Dr. Schonfeld and Rudel were the two speakers. What was interesting to me was the striking difference in response of human beings and primates that may not be so far removed from us in regard to their response to cholesterol feeding and changes in diet because it is clear that LDL metabolism in primates is dramatically affected by the diet and the size of the LDL particles are changed very strikingly and dramatically with dietary perturbations, whereas in humans it is difficult to demonstrate any changes in composition of particularly LDL, although there may be some minor changes. They are not very great, even though we can change the concentrations to some extent. So, I thought this was a particularly interesting difference and the reasons for this difference are not entirely clear, but it appears as though primates may be secreting a large amount of cholesterol with the VLDL, while this may not occur in humans.

SESSION VII SUMMARY AND DISCUSSION

DR.CHAIT: One general comment that I'd like to make which really underscores what several of the others have already said is that there obviously have been great strides in the physicochemical definition of heterogeneity and we are understanding much more about these heterogeneous particles. But we don't yet know enough about the physiological or metabolic heterogeneity. That is an area where a lot of us should be trying to concentrate our efforts over the next few years. Some specific comments relate to the genetics of the apoprotein B session VII. One of the things that is clear from that session is that there is something about the particles in the spectrum of disorders called familial combined hyperapobetalipoproteinemia that is different from those in other disease states. Therefore, it is encumbant upon any of us that are studying patients to try and learn a little bit more about our patient in order to characterize them with respect to the genetic disorder that they have and not necessarily to just put hypertriglyceridemia under one heading. We should think more carefully about what our patients have because this is going to determine quite a lot about what the lipoproteins are about.

We also heard about how use of monoclonal antibodies can show some genetic determinants of binding affinities to the antibodies. By that type of technique being pursued a little bit further one is also going to be able to show genetic variations that have metabolic consequences rather than just binding consequences to antibodies. In fact, we heard about this with respect to an ApoB in the pig that was metabolically distinct and genetically determined. I suspect that by using this type of approach we are going to find similar things in humans as well.

There was one thing that actually suggested some more homogeneity from that session. For the first time we heard that familial hyperApoB and familial combined are probably the same thing. So, that is one light at the end of the tunnel.

The disturbing thing that I found throughout the entire conference, the entire workshop was something we haven't actually discussed at all and that is heterogeneity of nomenclature. This is something that we can do something positive about, even at this stage now. A particular example would be familial combined and hyperApoB. Another example, of course, is the HDL nomenclature which in three talks back-to-back we heard three different systems of nomenclature with eleven different subclasses. I think we need to standardize the lipoprotein nomenclature.

SESSION VIII SUMMARY AND DISCUSSION

DR. ALBERS: Before discussing the HDL heterogeneity session i'd like to make some general statements. In the last couple of days a considerable body of evidence was presented to demonstrate structural heterogeneity and subclass heterogeneity of the major lipoprotein classes. Perhaps not as extensive, though certainly the evidence was considerable, that there is also functional heterogeneity. These distinct subpopulations must be taken into account in our future studies to evaluate the metabolism and functional role of these subpopulations.

Furthermore, although it is quite important we discussed very little, the inter-relationship between cell populations and the major lipoprotein classes. Also, for future studies it is necessary to further evaluate the genetic and physiological basis of the heterogeneity which we have discussed in the last few days. In addition, it is important to recognize from the data that was presented that the lipoprotein subpopulations can be significantly altered during sample handling and preparation. The limitations of our methods for identification and preparation of subpopulations must be recognized by investigators working in this area. Several particularly labile particles were noted and I'll just cite some examples. It is well known that ApoE is readily dissociated from B-containing lipoprotein particles. This must be considered when one wants to evaluate the functional role of the E/B particles in lipoprotein metabolism. Secondly, in regard to HDL heterogeneity, it was noted that the A1 from A1-containing particles without A2 is particularly labile and, in general terms, we have to be particularly cognizant of the labile proteins on the surface of lipoproteins particles such as LCAT and lipid transfer protein which functionally are very important, but somewhat more problematic in handling because of their relatively loose association with the lipoprotein surface. So, These problems will have to be addressed.

Therefore, in general, we can recommend that methods should be sought that minimally perturb the lipoprotein structure. Both methods that were discussed extensively, ultracentrifugation and affinity chromatography, have some limitations in this regard. We still have some improvements to make in addressing the problem of the proteins that are loosely associated with the surface, with low affinity.

Furthermore, the quantified subclasses of HDL, LDL and other lipoprotein species show considerable promise as potentially better predictors of atherosclerosis than our current predictors. However, this needs to be further evaluated in clinical applications. We therefore recommend that further efforts need to be made in the quantification of these subclasses.

DR. ROHEIM: Yesterday we were left with a frightening array of heterogeneous subclasses of HDL. Today, we have some idea or reasons why these subclasses exist. Dr. Barter's and Dr. Kunitake's work provided insight into why these subclasses may occur. Also, Dr. Johnson elucidated some factors which may influence cholesterol influx or efflux. But I would like to dwell on the factors, especially enzymes or proteins, present in the plasma which can influence the subclasses. We have to realize that when we treat a plasma sample it is not necessarily exactly the same as what's in the circulation. The message that I got is that we have to make very sure that as soon as we take the plasma we treat it in a very standardized way. So, I recommend that whenever an HDL presentation is given, the treatment of the plasma sample from the time of bleeding to the separation and cooling of the plasma and addition of different inhibitors to the samples should at least be noted. This would give us some information concerning how we can compare one set of data to another. This is a reasonably simple step to take.

A more complicated problem is that of different isolation methods. How do we obtain the most physiological lipoprotein fractions? There is no simple or single way to achieve this. We have to approach it in a multifaceted manner. I hope this conference gave us an impetus to try to integrate some of this data and to explore new avenues of investigation because this data is really very exciting. Not only do we have descriptive data, but I believe we are going more into the area of metabolism and mechanisms. That is where we should focus our attention in the future. I hope at the next meeting we will have much more functional and metabolic information.

DR. INNERARITY: The work that Alan Attie and Ron Krauss presented about the pig colony and the pig model has very great potential because some mutations have been worked out and they occur in LDL and ApoB. The problem is that Dr. Attie is a young investigator and I think he'll have very great difficulty in getting enough funds to do his research and to keep this colony going. So, I suggest that there be a recommendation that we don't want to lose this colony.

DR. KRAUSS: I certainly support that recommendation. The potential for looking at genetic models in a system that is so homologous, at least in the LDL, to the humans is really a very precious resource for all of us.

DR. GIANTURCO: Yes, I agree.

DR. ALBERS: A rabbit is also a good model and has been used more extensively because it reflects metabolism more similar to man than the pig does. And in the rabbit there are LDL polymorphisms that reflect differences in protein. So, we should also consider the rabbits a potential model to evaluate the mutations in ApoB and their effect on metabolism. These antibodies could be made available to those who would like to pursue this area of investigation.

DR. MELCHIOR: One area that we really haven't discussed at all in terms of heterogeneity are the differences between lipoproteins in the plasma compartment and those in interstitial fluid.

DR. ROHEIM: It is exceptionally important to realize what you see in the plasma might not necessarily be what the peripheral sites are in contact with. We have studied the interstitial lipoproteins and found distinct differences between them and the plasma lipoproteins. To date, we've studied mainly HDL which are very different. With respect to the post-absorptive and post-prandial changes, we don't have any idea at the present time how post-absorptive chylomicron remnants and VLDL remnants may or may not traverse through the endothelium into the interstitial space and what the physiological effects are. It is very important to determine if there is any transfer of these particles or not. We have just started a series of experiments on that issue. Another question to be addressed is the role of the free apoproteins which have an exceptionally important role. We have circumstantial evidence that one of the three apoproteins may be involved in reverse cholesterol transport. Parenthetically, I can say that in humans there may be free apoproteins other than previously discussed. Dr. Schonfeld says that under certain circumstances, free Al can be detected. In doing these studies how you treat your samples is very important because we have to realize that after the plasma is removed from the body it undergoes continuous changes. So, when we do all of these studies we have to be exceptionally careful to describe and prevent any post-physiological changes or changes which occur after the samples are taken.

DR. RUDEL: In the same vein as the comments that Dr. Deckelbaum and Dr. Roheim just made, interstitial fluid reflects the intravascular metabolism and maturation and changes about proteins that go on. There is still a lot of work to be done with regard to conversion of lipoproteins that leave the cells. Dr. Roheim's interstitial HDL may be a product of a secretory pathway that is not fully mature. Anybody that has worked on lipoproteins of perfused livers or interstitial fluid is struck by the total difference in composition of the lipoprotein particles that you encounter. They just don't look like the lipoproteins that we're used to isolating from plasma

samples. So, the aspect of maturation of lipoproteins is an important component of heterogeneity and probably not one that we heard too much about at this session, but is one that needs to be incorporated into the overall picture.

DR. GRUNDY: One question I would ask is why are we interested in all of this heterogeneity? If it is in relation to the possibility that different lipoproteins are more atherogenic than some others then we need to move into an area where we can begin to test this possibility. Right now, if you go down the list of lipoproteins, every single one has been claimed to be atherogenic. But, in fact, we don't really know whether any particular one is or isn't or whether one is more than another, whether IDL is more atherogenic than LDL. We simply don't know and we need to take one step more and try to develop some model systems that we can use to test these different lipoproteins for their potential effect on cellular uptake or macrophage uptake. With the animal models, Dr. Rudel's idea of trying to look at the relation between particular lipoprotein species and the development of atherosclerosis, although it may be little crude right now, is nevertheless a very good step in the direction that we need to move because when it comes to treating these conditions with different lipoprotein abnormalities we really don't know whether it is a good idea to treat them or not. We don't know whether we should treat people with high triglycerides or leave them alone. So, if we could begin to think about integrating this information in with the artery wall that would be a good step forward.

DR. SCANU: Our laboratory has been very intensively investigating protolytic enzymes. We have grown up with the idea that we like to inhibit all of the proteolytic enzymes. We draw blood and use all anti-proteolytic agents often with no rationale. And we don't know how far these proteolytic enzymes reach in fact. My concern is particularly relevant in this respect: we are starting to depart from ultracentrifugation which probably was the savior for us because some of the proteolytic enzyme could be bound to lipoprotein and displaced during ultracentrifugation. Now that we are going away from ultracentrifugation we may have problems under so-called physiological isolation conditions. If we take agarose-separated LDL--and use antibodies against specific enzymes, you can show beyond any doubt that a complex of alpha-1 antitrypsin, comigrates with LDL. My view is that we should be able to distinguish the bad enzymes from the good ones and realize that when we isolate lipoproteins and avoiding ultracentrifugation, we may not be exactly dealing with the same lipoproteins that are ordinarily called LDL and VLDL. I recommend that the role of proteolytic enzymes in lipoprotein heterogeneity should be explored. We started with LCAT. I remember always being told to use D_2O if you want the LCAT to stay on the lipoprotein and not to use salt. Well, the same applies to proteolytic enzymes which you can see floating or precipitated depending on what procedure is used.

DR. GIANTURCO: Dr. Bradley and I have done quite a bit of work on the binding of prothrombin to VLDL. We do find, as you suggested, that you can separate most of the prothrombin off by ultracentrifugation under the conditions under which we normally isolate lipoproteins in the presence of EDTA because prothrombin is a gamma carboxyl glutamate-containing protein that binds to the phospholipid surface through calcium bridges and by adding EDTA you can dissociate some, but not quite all of the prothrombin. The prothrombin that does associate with VLDL can be activated to thrombin on the surface and the thrombin can cleave ApoE and ApoB with profound effects on the binding of these VLDL to cell-surface receptors. Cleavage of E on hypertriglyceridemic VLDL abolishes binding to the LDL receptor while at the same time binding to a distinct macrophage receptor is enchanced. We believe this is due to the cleavage of ApoB by these proteases that associate with the VLDL.

Under different circumstances, different proteases could associate with different parts of the cascade, different lipoproteins. As Dr. Scanu has shown with the elastase and HDL, it is very important in terms of the routing of lipoproteins for metabolism and it may be a very key issue. I hope that a future workshop will be funded on the relationships among hypertriglyceridemics and atherogenesis. An important topic in such a workshop would be the role of coagulation factors associated with lipoproteins, particularly in hypertriglyceridemia where hypercoaguability appears to be prevalent in myocardial infarction.

DR. HAVEL: Proteolytic enzymes are important in a variety of ways. One other paradoxical problem has to do with the information of B-74 and B-26 which was not mentioned during the past three days. We now know how B-74 and B-26 are formed and under what conditions. At least in normal plasma, it is purely an in vitro phenomena and it requires exposure to cold. If you keep lipoproteins warm B-26 and B-74 are not formed. If you chill, they are. The mechanism has to do with kallakrein and its activation, the dissociation of kallakrein from its natural inhibitor.

The details were worked out by Andrew Gustus, John Kane and myself a year ago and will be published shortly. Sometimes steps we take to prevent things from happening actually cause them to happen. Also, there are increasingly valuable reagents becoming available in the form of monoclonal antibodies and I'd like to ask the people who work with monoclonals, who generate them, what their feeling is about repositories for monoclonals in this field? I can see certain ways in which that could be a big problem because they are proliferating exponentially. Probably two years ago there was less than a hundred; next year there may be a thousand. What are the practicalities? Clearly, the well-characterized ones can be extraordinarily valuable reagents to a variety of people. How is that best to be accomplished?

DR. SCHONFELD: I agree with Dr. Havel; monoclonal antibodies are proliferating. They are very useful and they should be shared much in the same way that cell lines in the cell repository are available to co-workers. Cell lines having to do with monoclonal antibodies should be similarly made available. The alternative to having cell lines might be to establish several places, perhaps funded by the NIH, that would make antibodies or ascites fluid or some form of the antibody available to workers much in the same way the pituitary agency made certain agents available. The practicality which must be considered is that several groups have made agreements with corporations which could potentially hamper some of the exchange of the cell lines. In my particular case, the agreement that I have with Mallinckrott is that the antibodies that were made under their support can be distributed to co-workers. They can't be sold commercially. Any commercial sales would be made by Mallinckrott. In terms of making antibodies available to people in the field,collaborators and co-workers, it is no problem. But it depends on the individual agreements with the corporations as to what the constraints would be. I agree with Dr. Havel that it would be very useful if there were ways of confirming epitope assignments in various labs because these are not trivial jobs to do. If the epitope assignments were well known, they would be even more useful.

Another item is the standardization of nomenclature for all of these lipoproteins and how one lipoprotein isolated by one system relates to another. I've heard a lot of people say over the course of the meeting that it would be advantageous if we could standardize nomenclature so that we know what we're talking about. Does anybody have any ideas on how that might be done? I know that a lot of people send samples to the Donner Laboratory for analytical ultracentrifugation and analysis by GGE and that's one way that a lot of people are beginning to communicate.

DR. KRUL: I've noticed that non-denaturing gels are becoming very popular. They are infiltrating methodology. Since a large fraction of the people who use these gels usually get them from Pharmacia who has good quality control in their labs then we are essentially using the same gels in our labs. If we can get the same lot that would be one way to standardize lipoprotein insolation. Dr. Nichols was apparently having the same problem as we were in getting the two to sixteen percent gels. But they've been proving very useful and someone from Dr. Ernie Schaefer's lab presented data at the Clinical Chemistry meetings in Chicago showing that LDL subfractions analyzed by the two to sixteen percent gel exhibited identifiable differences in coronary artery disease patients. So this is one easily accessible mode that allows one to look at a profile in GGE and it may be directly comparable to the profile from another group.

DR. GIANTURCO: That sounds very good for the IDL, LDL and HDL. It is perhaps a little bit more difficult for the VLDL range.

DR. BARTER: On the same question of separation, I think that we're going to be able to move in the IDL, LDL, HDL area with the non-denaturing gels into using two things. One, prestaining whole plasma with a lipid stain for 20 minutes running that on the fast gel system that exists on various gradients that can be developed. We can have a profile at four degrees two to three hours after collecting the blood sample. If that is done a lot of these other problems with storage are eliminated there is no reason why that procedure couldn't be standardized worldwide. We'd have a system then when all laboratories are not only using the same technique but they can then begin to develop the same terminology because one of the problems with terminologies is even just subtle differences in technique. So, what is an LDL-1 in one laboratory may be and LDL-1.5 in another laboratory.

DR. GIANTURCO: Would you suggest a hands-on workshop for something like this?

DR. BARTER: It would be very useful because it is potentially a very simple technique to use. It has obvious limitations because the lipid stain is not necessarily quantitative, but it will identify what fractions are there.

SPEAKER: That just brought a thought to my mind concerning relating the lipoproteins studied in the various animal systems to those in the human system. If we did set some sort of standardization for human lipoproteins, then would we animal people want to pick some normal human plasma and always run at a standard so we can jump back and forth between the animals and the humans. How do I relate what I'm seeing in the animal system to humans?

Dr. Barter: We've done a lot of animal work and had exactly the same problem which was the original reason why we stopped using fixed density in the ultracentrifuge. We wanted a continuum of some kind. So, now we use a mixture of ultracentrifugation, gel permeation chromatography, non-denaturing gel electropheresis and we don't make any predetermined ideas of what is an LDL, what is an HDL. We simply describe the continuum and that way, with the suggestion I'm making, the animal work would slot in perfectly without making prejudgments.

DR. KRAUSS: There is a related issue that I wanted to reemphasize regarding human genetic heterogeneity. We really didn't take it up as such in this conference. There is a whole universe of individuals out there doing restriction fragment-linked polymorphisms, defining genotypes in the population, some of which are ultimately bound to have ramifications in terms of lipoprotein heterogeneity. So, the issues that we've heard about regarding standardization and comparison with animal models

also relate to the ability to relate human genetic variance at the DNA level to given lipoprotein polymorphisms at the macromolecular level. I think that is an important area. More and more the field is going to be turning towards RFLPs as diagnostic and analytic tools and having that kind of a reference system to be able to look at the phsiological and pathological correlates of those DNA polymorphisms is going to be an extremely important link in the future.

DR. NESTEL: I just want to put in a plug more for sentimental reasons than any thing else for people working in man, the clinical investigators. We had a short session on lipoprotein kinetics. I'm mildly disappointed that more people are going out of the field than are coming into it. Although one of the pioneers felt it was too hard a number of years ago to continue. It really is a very daunting task. It is refreshing, on the other hand, that Dr. Havel who, when I first worked with him in Vale in 1960 said, "One mustn't touch that sort of thing. Physiology is the only thing. Lipoprotein modeling is too difficult" is now part of the group that's leading the field. But what I really wanted to say was that lipoprotein heterogeneity or heterogeneity of metabolic processes possibly started ahead of any other area. If one looks back to Dr. Waldo Fishers' contribution; or to Baker's early 1960 papers where he had countless pools in the plasma to explain fatty acid and also VLDL metabolism this approach was looked on as sort of a mathematical concept which may not have any phsiological counterpart.

The talks we had yesterday showed quite clearly that the lipoprotein kineticists are a very flexible group. The number of models, the number of compartments and so on are increasing all the time. They are taking cognizance of the fact that was brought up earlier that some of the lipoprotein remodeling takes place not in the plasma, but outside of the plasma and there are lipoproteins that move to the liver and interact with enzymes. They reenter the circulation as Dr. Zech pointed out yesterday. It is a very daunting task because, in the face of all of the in vitro data that we've had in the last two days, the compositional and structural heterogeneity of lipoproteins, it is remarkable that there is anybody that wants to engage in this in vivo game. But I suggest that it ought to be supported very strongly in this country.

DR. ROHEIM: One of the problems in standardization is to obtain reliable high molecular weight standards. If we had proper standards, then we could use whole media and the molecular weight could be calculated on that basis. We tried latex beads and whatever else was available but didn't succeed very well. So, if we can get some help or some direction, that would be a very useful contribution for a large number of lipoprotein people. Second, Dr. Havel's comments on the repository of cell lines is an excellent idea but I would very much prefer monoclonal cell lines in the repository. This question came up in the NHLBI Apoprotein Quantitation Workshop, held in 1982. The question is whether NHLBI has the financial resources. For such a project. It would be very important, but alternatively, if we cannot have a repository, it would be beneficial to compile a list of available well characterized monoclonal antibodies which could be distributed to the field. So, if somebody wanted to do a specific experiment, they would not have to go through the entire literature but would just have to look into the available cell lines. That might be a simplified and more economical way to make those cell lines accessible to the public and I assume that everybody would make it available for all investigators.

DR. PACKARD: We're getting quite happy about IDL and LDL and HDL. The VLDL still seems to be a problem. Probably Frank Lindgrens' technique of cumulative flotation ultracentrifugation is the best defined technique that we have. That technique could be applied to VLDL since we are not likely to get polyacrylamide gels to separate VLDL in sizes. It would all fall apart. And a lot of people in this room have used

it. So we could standardize that technique. One aspect of that technique that might be further investigated when trying to define density cuts and putting things together or slicing things apart is to get good hydrated densities to do the calculations with. Is it possible for Dr. Krauss' lab to get better hydrated densities for some of those particles when you start chopping it up? Secondly, if we had a computer program into which the S_f rates required for a certain individual or a certain experiment could be fed and out would come the run times, the centrifugation times, and the speeds. That would save a lot of work. I have done those calculations a few times and it is fairly laborious.

DR. KRAUSS: The answer to both issues is, yes. Better systems could be developed, better data could be developed that would be more precise than using the literature. Frank Lindgren is still very interested in pursuing this matter and I will take that message back to him as a potential area of development.

DR. SCHONFELD: I'd like to second the idea of defining ultracentrifiguation conditions and making it easy for people to calculate run times. It would be very helpful, particularly for people who are getting started. But everything that has been said about the monoclonal antibodies and the repositories is equally applicable to the cDNA and genomic DNA clones that are becoming available. They will be just as useful reagents for certain kinds of studies as the monoclonal antibodies are. That raises another issue of heterogeneity. There is a tremendous heterogeneity between labs as to their generosity with the clones. Some people are very generous and will send as many clones as they have very soon after they become available. Not everyone will do that. Since most of the clones are generated with public funds the refusal to share clones really goes against the tradition in research of sharing and being generous with other workers.

DR. INNERARITY: What is the policy of the journals in our field regarding antibody and cDNA? If you publish an article and use a monoclonal antibody, do you have to make that antibody available?

DR. MELCHIOR: Many of the journals now are stating as their editorial policy the requirement that if you are publishing on a monoclonal antibody or a hybrid cell line then you have to make these reagents available to colleagues in the field in a reasonable amount. Otherwise, it is a useless reagent. No one can confirm that work. It may pay to have a more unified system among the journals so that everyone is clear what the policy is and regardless of which journal you publish in, you are following the same rules.

DR. HAVEL: One suggestion did come along separate from a repository and strikes me as being potentially a lot simpler. We are dependent upon the generosity of colleagues for providing these kinds of resources to us. Finding out about them isn't always so easy. I wonder if some kind of central data bank not just for monoclonals, but for other things such as clones, maybe even cell lines in some cases, could be a very useful thing. That wouldn't be nearly so expensive or difficult to implement. It would have to be voluntary. I don't know of any way to enforce that, but it might be worthwhile exploring mechanisms by which that could be done and seeing if something might be brought forward.

DR. MELCHIOR: I'd like to address that point again. Although people should make these cell lines, antibodies and clones available, it can really become a major part of their program just to start making monoclonal antibodies or cell lines available.

Barbara Knolls was actually inundated for over a year with requests for the Hep-G2 cell line that she developed. Although some people want to be very generous, if it happens to be something popular, it just can't be done.

DR. LIPPEL: I'd like to thank everybody who participated in the workshop, especially Sandra Gianturco for all the effort she put into it. We intend to publish a summary in "Arteriosclerosis" in May - June 1987 and the complete proceedings within the next year. Thanks once again.

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